# ANNUAL REVIEW OF BIOCHEMISTRY


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## PREFACE

With the post war period of rehabilitation slowly in progress the resumption of international co-operation in the things of the mind is both hopeful and encouraging. The background is a world that stubbornly persists in attempting to be more than one world. The foreground is the unity and potentialities of Science as a unifying influence: an adequately verified discovery reported from Addis Ababa is just as meaningful as one from anywhere else; and the mind of man functions through the same kind of a cerebral cortex in the East as in the West.

It is good to learn that journals of original publication, suspended during the war, are gradually being revived. The flow of literature from country to country is rapidly being resumed. International conferences of scholars are again being held and visits of scientists from country to country are again becoming possible. All these, and other, evidences of cultural exchange and international amity in the world of scholarship are of great significance. In this present volume of the *Annual Review of Biochemistry* it is a pleasure to report that about 25 per cent of the reviews were written by our colleagues abroad, an encouraging approach to the prewar average of about 50 per cent.

We regret that a review on the nucleoproteins which we had hoped that Professor E. Hammarsten might prepare did not materialize, likewise one on the waxes by Professors Chibnall and Piper. Publication of papers on chemistry of penicillin will become permissible in September, 1947. This will allow us at last to include the critical review which we have long hoped to publish, now scheduled for inclusion in Volume XVII.

We are continually reminded by our colleagues abroad of their very great need for reprints of current papers and of others published during the years of the war. These can be especially useful to the contributors selected for forthcoming volumes of the *Annual Reviews*. In order to encourage the dispatch of reprints we present on the next three pages the names and addresses of those who are expected to participate in authorship of forthcoming volumes of the three Reviews.

We are, as ever, extremely grateful to the many who have con-

tributed in the authorship of the present volume. The preparation of reviews in which the author critically evaluates the research of the preceding year or biennium is always a difficult and unhappy task. In expressing our thanks as an Editorial Committee we feel that with propriety we are also conveying the gratitude of the many readers of the Review. It is also a pleasure to express our appreciation to the George Banta Publishing Company for their cordial co-operation in the printing and manufacture of the volume at a time when the entire printing industry was beset with manifold difficulties. Finally, to our editorial assistants and the office staff generally we wish to express more than a word of thanks for their help on the present volume.

H.J.A. J.M.L.
H.J.D. H.A.S.
D.R.H.

# TOPICS AND AUTHORS

## ANNUAL REVIEW OF MICROBIOLOGY
## VOLUME II (1948)

Genetics of the Fungi—Lindegren, C. C., Department of Botany, Washington University, St. Louis 5, Missouri

Metabolism of Microorganisms—Gunsalus, I. C., Laboratory of Bacteriology, New York State College of Agriculture, Cornell University, Ithaca, New York

Metabolism of Protozoa (Malarial parasites in particular)—Moulder, J. W., Department of Bacteriology and Parasitology, The University of Chicago, 5724 Ellis Avenue, Chicago 37, Illinois

Growth Factors for Microorganisms—Koser, S. A., Department of Bacteriology, The University of Chicago, 5724 Ellis Avenue, Chicago 37, Illinois

Nitrogen Fixation—Virtanen, A. I., Biochemical Institute, Helsinki, Finland

Antibiotics—Bailey, J. H., and Cavallito, C. J., Sterling-Winthrop Research Institute, Rensselaer, New York

Mode of Action of Chemotherapeutic Agents—Hotchkiss, R. D., The Rockefeller Institute for Medical Research, 66th Street and York Avenue, New York 21, New York

Complement—Ecker, E. E., Institute of Pathology, Western Reserve University, 2085 Adelbert Road, Cleveland, Ohio

Nature of Antibodies—Pauling, L., and Campbell, D. H., Department of Chemistry, California Institute of Technology, Pasadena, California

Inheritance of Immunity in Animals—Gowen, J. W., Department of Zoology, Iowa State College, Ames, Iowa

Yeasts—Mrak, E. M., Division of Food Technology, University of California, Berkeley 4, California

Microbiology of Soil—Smith, N. R., Bureau of Plant Industry, Division of Soils, Fertilizers & Irrigation, U. S. Department of Agriculture, Beltsville, Maryland

Pathogenic Streptococci—Dingle, J. H., and Rammelkamp, C. H., Jr., Department of Preventive Medicine, Western Reserve School of Medicine, Cleveland, Ohio

The Neurotropic Viruses—Schultz, E. W., Department of Bacteriology, Stanford University, California

Spirochaetes—Davis, G. E., Rocky Mountain Laboratory, U. S. Public Health Service, Hamilton, Montana

The Microbiology of Water and Sewage—Smit, J., Landbouw Hoogeschool, Wageningen, Holland

Chemical Disinfectants—Wyss, O., Department of Bacteriology, University of Texas, Austin 12, Texas

Bacteriophage—Gratia, A., Univ. de Liege, Laboratoire de Bacteriologie, 1, Rue des Bonnes, Villes, Liege, Belgium

Bacteria as Plant Pathogens—Burkholder, W. H., Department of Plant Pathology, Cornell University, Ithaca, New York

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## ANNUAL REVIEW OF BIOCHEMISTRY
## VOLUME XVII (1948)

Biological Oxidations and Reductions—Weil-Malherbe, H., Department of Physiology, Medical School, Kings College, Newcastle-on-Tyne, England

Nonoxidative Enzymes—Sumner, J. B., Department of Biochemistry, Cornell University, Ithaca, New York

Chemistry of the Carbohydrates—Prins, D. A., and Jeanloz, R. W., 3141 Maplewood Ave., Montreal 26, Canada. Institute of Experimental Medicine and Surgery, University of Montreal, Quebec, Canada.

Chemistry of the Immunopolysaccharides—Haworth, W. N., and Stacey, M., Department of Chemistry, The University, Edgbaston, Birmingham 15, England

Structural Studies with X-Rays—Crowfoot, D., Laboratory of Chemical Crystallography, Oxford Museum, Oxford, England

Chemistry of the Lipids—Folch-Pi, J., McLean Hospital, Waverley, Massachusetts, and Sperry, W. M., New York State Psychiatric Institute and Hospital, 722 West 168th Street, New York, New York

Chemistry of Proteins and Amino Acids—Pedersen, K. O., Institute of Physical Chemistry, University of Upsala, Upsala, Sweden

Nucleoproteins, Nucleic Acids and Derived Substances—Chargaff, E., and Vischer, E., Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York

Carbohydrate Metabolism—Vennesland, B., Department of Biochemistry, University of Chicago, Chicago, Illinois

Lipid Metabolism—Chaikoff, I. L., Department of Physiology, University of California, Berkeley 4, California

Metabolism of Proteins and Amino Acids—Allison, J. B., Department of Biochemistry, Rutgers University, New Jersey

Detoxication Mechanisms—Bodansky, O., Department of Pharmacology, Cornell University, Medical College, 1300 York Avenue, New York 21, New York

Clinical Applications of Biochemistry—Madden, S. C., and Zeldis, L. J., School of Medicine, Emory University, 36 Butler Street, S.E., Atlanta 3, Georgia

Chemistry of the Hormones—Gallagher, T. F., 16105 28th Ave., Flushing, New York, New York

Chemistry of the Vitamins—Oser, B. L., Food Research Laboratories, Inc., 48-14 33rd Street, Long Island City, New York

Clinical Aspects of Vitamins—Spies, T. D., Nutrition Clinic, Hillman Hospital, 1901 6th Avenue, Birmingham, Alabama

Nutrition—Sinclair, H. M., Oxford Nutrition Survey, Summertown House, Oxford, England

Ruminant Nutrition—Phillipson, A. T., and Elsden, S. R., Department of Physiology, The University, Cambridge, England

Chemistry of Neoplastic Tissue—Rusch, H. P., and LaPage, G. A., McArdle Memorial Laboratory, University of Wisconsin, Madison, Wisconsin

Biochemistry of the Natural Pigments—Lederer, E., Institut de Biologie Physico-Chimique, 13 Rue Pierre-Curie, Paris, (5e), France

Terpenes—Mirov, N. T., California Forest Experiment Station, University of California, Berkeley 4, California

Chemistry of the Alkaloids—Dawson, R. F., Department of Botany, Columbia University, New York, New York

Photosynthesis—Wassink, E. C., Biophysical Research Group, Physisch Laboratorium der r. u., Bijlhouwerstraat, 6, Utrecht, Netherlands

Mineral Nutrition of Plants—Burström, H., Botaniska Laboratoriet, Lunds Universitet, Lund, Sweden

Growth Substances in Plants—Zimmerman, P. W., Boyce Thompson Institute, 1086 Broadway, Yonkers, New York

Bacteria—Gunsalus, I. C., New York State College of Agriculture, Cornell University, Ithaca, New York

Penicillin—Chain, E., Sir William Dunn School of Pathology, South Parks Road, Oxford, England

# TOPICS AND AUTHORS

## ANNUAL REVIEW OF PHYSIOLOGY
## VOLUME X (1948)

# ERRATA

Volume X, page 512, equation (11):

*for*
$$C_A = \frac{E_{2B} \log \dfrac{I_{2'}}{I_2} - E_{1B} \log \dfrac{I_{1'}}{I_1}}{E_{1A}E_{2B} - E_{2A}E_{1B}}$$

*read*
$$C_A = \frac{E_{2B} \log \dfrac{I_{1'}}{I_1} - E_{1B} \log \dfrac{I_{2'}}{I_2}}{E_{1A}E_{2B} - E_{2A}E_{1B}}.$$

Volume XI, page 576:

Interchange Structures VIII and IX.

Volume XIII, page 45, line 9:

*for* increase *read* decrease.

Volume XV, page 367, line 13:

*for* reversed at 64°C. *read* reversed after 64 hours.

Volume XV, page 508, lines 12 and 13:

*after* Weil and Johnson (56) *delete* indicating a half-life of about 70 hours.

Volume XV, page 508, line 24:

*after* animal (58) *insert* indicating a half-life of about 70 hours.

# CONTENTS

# BIOLOGICAL OXIDATIONS AND REDUCTIONS[1]

By L. Michaelis
*Laboratories of The Rockefeller Institute for Medical Research, New York, New York*

## Oxygen Uptake; Respiration

Jayle & Schwob (1), working on tissue slices of kidney, liver, heart, and brain in the Warburg apparatus, claim that serum proteins exhibit a stimulating effect on respiration. When added to glucose-free Ringer solution they promote oxygen consumption beyond that obtained in control experiments with glucose-Ringer solution. The authors rule out the possibility of oxidative deamination of amino acids or participation of lipids and ascribe this function to the carbohydrate content of the serum proteins. They believe this result to be a support of Lépine's hypothesis of the existence of a virtual utilizable sugar reservoir in the blood.

In a well-dialyzed suspension of pigeon brain (2), respiring *in vitro*, addition of fumarate increases the oxygen uptake. In the presence of arsenite, pyruvate is formed. The reaction is catalyzed by inorganic phosphate but strongly inhibited by malonate. It is not affected by adenine nucleotide. Anaerobically, with methylene blue as acceptor, the results are similar. *l*-malonate behaves similarly to fumarate.

Conway & Breen (3) have studied the oxygen consumption of bakers' yeast cells in which potassium ion was displaced by ammonium ion by means of culturing the yeast in a suitable medium. The rate of fermentation of glucose by ammonia-yeast was about 40 per cent that of ordinary potassium-yeast; growth was at first slow, later increasing rapidly, but the cells were smaller. Addition of very little potassium changed the behavior to that of potassium-yeast. The oxygen uptake of ammonia-yeast in the resting state was considerably higher than in potassium-yeast, probably because ammonia served also as a nitrogen source. The presence of carbon dioxide was essential for the interchange of potassium and ammonium ions which suggests that formation of carbaminate is involved in the penetration of ammonium ion into the cells.

Atabrine inhibits the oxygen consumption of rat liver, brain, and kidney slices (4). Oxidation of glucose, lactate, pyruvate,

[1] This review covers the period from July 1945 to November 1946.

malate, and citrate is inhibited, but not that of succinate. Atabrine also inhibits *d*-amino oxidase. Probably it interferes with the yellow enzyme system.

Spinach leaves contain an abundant amount of polyphenol oxidase (5). This enzyme seems to be the "terminal" oxidase. The rate of respiration can be increased by dihydroxyphenylalanine which is a substrate for that enzyme.

*Neurospora crassa* and *N. sitophila*, according to Giese & Tatum (6), have a $Q_{O_2}$ of from ten to fifty-five c.mm. of oxygen per mg. dry weight per hour, depending on age and state of nutrition. Morphological mutants which grow slowly respire at the same rate. In wild types starved for sucrose the $Q_{O_2}$ may fall as low as five increasing by 340 per cent on addition of sucrose. Cultures starved for thiamine alone show only a small decline, and when starved too long, may respond only feebly to sucrose. Vitamin mutants may show lower $Q_{O_2}$ on starvation in respect to both sucrose and vitamin. The respiratory quotient is usually $>1$ in healthy cultures, close to 1 in vitamin deficient ones, and $<1$ in cultures starved for sugar. From the effect on properly starved cultures it may be concluded that *p*-aminobenzoic acid, pantothenic acid, and pyridoxine function in the synthesis of the protein parts of the enzymes. The same authors (7) have studied the influence of sulfanilamide on the respiration of *Neurospora*. It has no influence either on healthy or starved wild cultures or on *p*-aminobenzoic-less strains. Deficiency of thiamine does not make the respiration more sensitive to the sulfa drug. Mycelia, starved in the presence of sulfanilamide, when supplied with sugar respond even better than normal mycelia. The authors conclude that sulfanilamide has a preservation effect on the respiratory structure of the cell, perhaps by inhibition of the destruction caused by enzymatic proteins during the starvation.

Since the change of oxygen uptake due to cyanide poisoning is an important and frequent subject of investigation, two papers (8, 9) should be mentioned which deal with the theoretically difficult and experimentally important problem of establishing and maintaining a known and constant hydrocyanic acid pressure in the respiration vessel.

According to Stadie & Haugaard (10) mice under eight atmospheres of oxygen rapidly develop severe symptoms. They conclude

that the acute phase of oxygen poisoning is not a hyperoxic anoxia. Haugaard (11) suggests that this effect may be in some way correlated with the effect of oxygen on sulfhydryl groups of certain enzymes.

Nizet (12) describes a gasometric determination of the oxygen saturation of blood, based on the fact that phenylhydrazine reacts with oxyhemoglobin and develops one volume of nitrogen to one volume of oxygen.

The action of azide on respiration, luminescence, and growth in luminescent bacteria has been studied by Giese (13). Endogenous respiration is less sensitive to azide than that in the presence of nutrients. The latter depends on the particular substrate; it is least affected by glycerol, more by peptone, succinate, pyruvate, or glucose. Very low concentrations of azide sometimes increase respiration. The inhibition by azide was under no condition more than 90 per cent. Luminescence is almost entirely quenched, and growth is even more sensitive.

## General Theory of Oxidative Catalysis

Haurowitz (14) discusses the general nature of biological oxidation catalysis. The fundamental hypothesis is that a catalyser-substrate complex is formed in which a shift of electrons takes place intramolecularly. He discusses especially the catalytic oxidative effect of hemin. Its alleged oxidative effect on aldehyde is shown to be due not to hemin, but to the pyridine contained in the solvent used in the experiments. The catalytic effect of hemin toward the oxidation of hydrogen sulfide is exerted not only by hemin, but also by cobalt sulfate or nickel sulfate, which makes it doubtful whether the shift from ferrous to ferric iron is essential for the catalysis. The catalytic effect of hemin on oxidation of linoleic acid occurs only in a heterogeneous medium, and not when the fatty acid is in the state of a true solution, as in alcohol, pyridine, etc. So, only the interface is catalytically active. The catalytic action of hemin is ascribed to an inductive effect upon the groups within the substrate capable of reaction, and consists in an intramolecular transfer of electrons, which sometimes gives rise primarily to a free radical. The experiments with catalytic oxidation of hydrogen sulfide suggest as the primary reaction not the formation of a free sulfhydryl radical, but the one-step, bivalent reaction,

$H_2S + O_2 \rightarrow S + H_2O_2$. The system hemin-linoleic acid is a protein-free catalytic system, which oxidizes itself catalytically: both hemin and linoleic acid are oxidized. Analogous protein-free catalytic systems affecting carbohydrates or proteins, however, have not yet been shown to exist. So it appears doubtful whether hemin may be justly considered as a model for physiologically important oxidative catalysts.

The reviewer wishes to add a critical remark with respect to one item, seemingly concerned with just a detail but in fact of fundamental significance. Haurowitz claims that in the oxidation of hydrogen sulfide only a one-step, bivalent oxidation can be noticed, and not an oxidation in two successive, univalent steps involving a free sulfhydryl radical. Practically speaking, this may be granted, since the free sulfhydryl radical can never be detected. However, the oxidative formation from hydrogen sulfide of a disulfide (and polysulfides), such as hydrogen disulfide ($H_2S_2$), is understandable only on the assumption of the transient formation of the sulfhydryl radical, just as the oxidation of cysteine (RSH), to cystine (RSSR), is understandable only on the assumption of a transient free SR radical. It may be true that the overlapping of two univalent steps in a bivalent oxidation is often so complete that it is merely an academic dispute whether one should speak of a one-step, bivalent process, or of a two-step process in the limiting case, namely with complete overlapping of the two steps. However, it is not an academic dispute but a statement based on experience, if one claims that all processes which appear to take place in one bivalent step require a very high activation energy. This is equivalent to saying, whenever a univalent step requires much energy, that the bivalent step requires a high activation energy. The function of an oxidative catalyst is to lower the activation energy. Haurowitz (14) and the reviewer (15, 16) agree in the hypothesis that a complex compound is formed from an enzymatic center (the apoenzyme, a specific protein), the oxidizing agent (which is often represented by a prosthetic group such as flavin), and the substrate to be oxidized, and that the interaction of the last two is facilitated by their suitable orientation and distortion ensuing from their adsorption on the protein surface. The specificity resulting from the nature and shape of the protein surface is the same problem, not yet solved, both in enzymology and immunology.

## Cytochrome

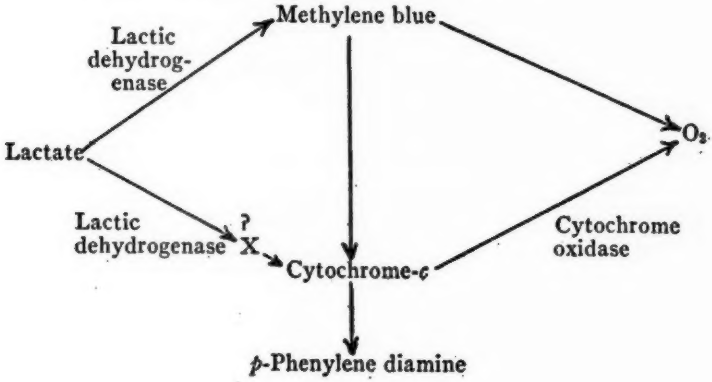
Cytochrome-*c* prepared from heart muscle or yeast contains 0.34 per cent iron and is also electrophoretically homogeneous. Theorell & Akeson had noticed that at a pH very slightly above 10.6, the isoelectric point, the red cytochrome slowly moves toward the anode, while a colorless boundary moves more rapidly. The more slowly moving colored fraction has 0.43 per cent iron. Keilin & Hartree (17) describe a more convenient method for preparing this iron-rich cytochrome. First, the usual cytochrome with 0.34 per cent iron is prepared from heart muscle. From this cytochrome which contains 0.43 per cent iron is prepared by saturation with ammonium sulfate, bringing to pH 10 with ammonia, discarding the precipitate, dialyzing against 0.5 per cent potassium chloride, evaporating in a cellophane bag by means of an air current to a small volume, and again dialyzing against 0.5 per cent potassium chloride. This cytochrome contains 0.43 per cent iron and shows only one moving boundary (at both pH 7.3 and 10.7). The colorless protein fraction could not be prepared successfully in a pure condition. The two forms of cytochrome-*c* are functionally indistinguishable. It is assumed that the colorless protein forms a part of cytochrome-*c* as it exists intracellularly and is not an accidental contaminant.

Bach *et al.* (18) have described a new kind of cytochrome in yeast, designated as cytochrome-$b_2$ which, although distinctly different as to its absorption band from any other cytochrome, is present in too low a concentration in yeast to be directly recognizable spectroscopically. It plays an essential role in the lactic acid dehydrogenase of yeast. The activity of yeast extracts with respect to this dehydrogenase runs parallel to the concentration of this cytochrome. Possibly another additional factor is essential for the activity, although it has not yet been identified or isolated.

In general, one can distinguish three classes of dehydrogenases: (*a*) those depending on the presence of pyridine nucleotides (coenzyme I and II). Through the interaction of these coenzymes, and only thereby, they react with flavoproteins, later on with the cytochrome system and then with oxygen; (*b*) those which, without coenzyme interaction, react directly with flavins, then further, either directly or with intermediation of diaphorase and cytochrome with oxygen; and (*c*) the "cytochrome-reducing dehydrogenases," which readily reduce cytochrome-*c*, without

the intervention of any known soluble coenzyme, and also reduce methylene blue directly. This action may not be direct either, but the intermediators, if any, are not extractable, known coenzymes. Dehydrogenases of class *a* have been obtained in the crystalline state. They contain no colored prosthetic group. Several enzymes of class *b* have been approximately purified. None of type *c* has been purified or isolated so far. They are usually attached to the insoluble part of the cell structure. Examples are muscle succinic acid dehydrogenase, lactic acid dehydrogenase of yeast (not that of muscle), the insoluble glycerophosphate dehydrogenase of animal tissues. In contrast to other dehydrogenases of this type (*c*), the lactic acid dehydrogenase of yeast is freely soluble and for this reason was made the subject matter of the present paper. Although it had been extracted previously by Bernheim and found also in autolysates, it has never been concentrated to any appreciable extent. Complete isolation has not been successful even yet, owing to the fact that the enzyme becomes more labile the more it is purified. The present stage of purification depends on the fact that its stability is appreciably increased in the presence of its substrate (lactate). It is then relatively resistant to heat so that much protein admixture can be removed by heat coagulation without losing too much of the active enzyme which remains in solution. It is adsorbed by calcium phosphate at pH 5 to 6, and can be eluted with phosphate (pH 5 to 6), only in the presence of much ammonium sulfate. Ultrafiltration may then be applied. The filtrate shows an absorption band at 5565 Å ($\alpha$-band) and a weaker one at 5300 ($\beta$-band). The $\alpha$-band lies between those of cytochrome-*a* and *c*. This cytochrome-$b_2$ resembles cytochrome-*b* in so far as it is slightly autoxidizable. It does not react with carbon monoxide. If the preparation be frozen in liquid air, the $\alpha$-band is resolved to a doublet. Its hemin component is a protoporphyrin, as in the case of cytochrome-*a* and *c*. In a Manchester yeast, this cytochrome corresponded to 0.0040 mg. hemin per cc. The molar absorption coefficient is approximately the same as for other cytochromes. It is considered to be the lactic acid dehydrogenase, or at least an essential component of it. Its turn-over number is calculated as 2900 per minute. (In succinic dehydrogenase it is 1400, and in living yeast 3800; that of diaphorase from 1000 to 8000, according to concentration.) There is no evidence that an additional factor is necessary to enable the system to react with cytochrome-*c*.

The following scheme represents its function:




In this scheme, "lactic dehydrogenase" is cytochrome-$b_2$, possibly in combination with an unknown factor.

According to Friedenwald and his associates (19), the addition of cytochrome-*c* to a solution containing oxidized epinephrine and ascorbic acid increases considerably the oxygen uptake and the disappearance of ascorbic acid. The authors rule out the posibility that this effect might be due to an incomplete elimination of cytochrome oxidase or of metal ions from the cytochrome-*c* preparation. Ascorbic acid cannot be replaced in this reaction by other reducing agents such as hydroquinone, or catechol, which all reduce cytochrome-*c*. A specific combination of oxidized epinephrine and ascorbic acid with cytochrome, enhancing the possibility of electron transfer at the adsorbing surface of the protein, is postulated.

Keilin & Hartree in previous papers (1937 and 1939) had concluded that cytochrome-*c*, unlike other ferriheme compounds, did not form a cyanide complex. This has become doubtful (Potter, 1941). Horecker & Kornberg (20) have taken up this problem and found that this complex does exist. The failure to discover it before is due to the fact that the shift in the absorption band is too small to be detected by direct visual spectroscopy. The interaction of cytochrome-*c* and cyanide is slow and requires a relatively high concentration of cyanide ions. Though the displacement of the main band is small, the cyanide complex can be readily recognized by the fact that a band of ferricytochrome-*c* at 6925 Å (not

before known) disappears after the reaction with cyanide. The slowness of the reaction permits kinetic studies of the reaction spectrophotometrically with a light filter transmitting only wave lengths from 5350 to 5700 Å. Under these conditions the optical density is always proportional to the concentration of ferricytochrome-*c*; but for reduced cytochrome-*c* this is true only for concentrations $<2.0\times10^{-5}$ *M*.

Cyanide-ferricytochrome-*c* is not appreciably reduced by succinic dehydrogenase, but readily reduced by sodium hydrosulfite. The rate of interaction of ferricytochrome-*c* and potassium cyanide, provided the latter is measured in terms of the free cyanide ion, is that of a first order reaction. The kinetic reaction constant, $K'$, is 550 at 24°C. and 3810 at 37°:

$$K' = \frac{2.303}{[CN^-]} \times \frac{d \log_{10} [\text{ferricytochrome-}c]}{dt}.$$

From this the activation energy is computed to be 26,100 calories per mole. The equilibrium constant $K$, corresponding to the reaction cyanoferricytochrome-$c \rightleftharpoons$ ferricytochrome-$c + CN^- + 6400$ cal. ranges from 6.3 to 2.0 over a temperature range from 5° to 37°C. The sluggishness of the interaction of cyanide ion and ferricytochrome-*c* makes it most unlikely that the poisonous effect of cyanide on respiration can be attributed, even in part, to this reaction. When cyanide ions combine with cytochrome oxidase, ferricytochrome-*c* is liable to be rapidly reduced so that there is no time for its interaction with cyanide.

Cytochrome oxidase and succinic acid dehydrogenase are closely linked, forming together the "succinic oxidase system." They are structurally, also tightly, attached to the same particles of the cell [although recently they have been separated: cytochrome oxidase has been separated by Haas, and succinic dehydrogenase recently by Hogeboom (67)]. The development of the two enzymes was followed (21) during the development of the chick embryo, from twenty-five hours to twenty-one days, and proved to be different. The cytochrome oxidase was more abundant than the other in the early stages, then dropped to a constant level from the fifth day on.

The action of various inhibitors on various oxidative systems involving cytochrome-*c* was compared in Elvehjem's laboratory

(22). The enzymatic oxidation of ascorbic acid, glutathione, and succinate belong in this category. The manner in which various inhibitors acted in these three cases was investigated. The following inhibitors were chosen: cyanide, azide, diethyldithiocarbamate, 8-hydroxyquinoline, thioglycolate, hydroxylamine, pyrophosphate, 2,4-dinitrophenol, and *p*-nitrophenol. With glutathione as substrate, inhibition was observed with those of the quoted chemicals known to combine with copper; for ascorbic acid, those reacting with iron, and for succinic acid as substrate, those reacting with either iron or copper. At least two types of inhibitors exist the properties of which may be attributed to their affinity either for copper or for iron. This finding suggests that the interaction of cytochrome-*c* with a substrate is of more complicated a nature than has been assumed previously.

When the action of cytochrome oxidase is partially inhibited by cyanide, this inhibition can be counteracted by methemoglobin, which forms a cyanide complex (23) and thus brings about a competition of the cyanide for cytochrome oxidase and methemoglobin. This competition was studied spectrophotometrically by measuring the ratio of reduced to oxidized cytochrome-*c* in the system. The author discusses the therapeutic implication for treatment of cyanide poisoning.

Proger & Dekaneas (24) recommend the use of cytochrome-*c* for the treatment of anoxia, starting from the supposition that even in severe anoxia there is still a considerable oxygen content in the blood, the utilization of which can be augmented by increasing the oxygen activating enzymes. Among these, cytochrome-*c* appears to be the limiting factor, whereas cytochrome oxidase is not. Cytochrome-*c* is not toxic and, although a protein, not antigenic.

Localization of oxidative enzymes within the cell was attempted by Schneider (25) and by Hogeboom *et al.* (26). The former finds succinic dehydrogenase and cytochrome oxidase associated with the larger granulae, the mitochondria. The major part of the activity of these enzymes, and also of adenosinetriphosphatase is in these granules. Desoxypentose nucleic acid was found only in the nuclear fraction of the cell sediment. Pentose nucleic acid was found in the the large granules, in the nuclear sediment and, most of all, in the unfractionated residue. Lipoid phosphorus was found in higher concentration in the large granule frac-

tion, but most of it in the unfractionated residue. Hogeboom *et al.* (26) also found cytochrome oxidase and succinic dehydrogenase associated with the sedimentable granules of relatively large size (0.5 to 2$\mu$ diameter). The smaller granules (60 to 150 m$\mu$ diameter) showed little enzymatic activity, the residue none.

## Catalase and Peroxidase

Keilin & Hartree (27) throw doubt on the hypothesis that the essential physiological function of catalase is to destroy hydrogen peroxide which is primarily formed whenever oxygen is the hydrogen acceptor in an enzymatic oxidative reaction. They attribute to catalase essentially another function. These authors had shown in 1936 that a "primary oxidizing system" (such as xanthine oxidase plus hypoxanthine; or *d*-amino acid oxidase plus amino acid) which reduces oxygen to hydrogen peroxide, in the presence of catalase and on addition of ethanol, develops no hydrogen peroxide but instead brings about an equivalent oxidation of the alcohol. So catalase, instead of destroying hydrogen peroxide, activates it for the oxidation of alcohol. Instead of the hydrogen peroxide developed by the enzymatic system, also barium peroxide or cerium peroxide or a very weak solution of hydrogen peroxide, but not hydrogen peroxide at high concentration, performs the same oxidation of alcohol in presence of catalase. It appears as though "nascent" hydrogen peroxide is the oxidizing agent for alcohol. The idea that this nascent hydrogen peroxide is different from ordinary hydrogen peroxide (say an excited molecule or a free radical) is discarded; rather the efficiency is based on the low concentration of hydrogen peroxide at any given time. In this case, it is utilized for the oxidation of the alcohol instead of being decomposed to oxygen plus water. An interesting case arises when the primary oxidative system is represented by the system: xanthine oxidase plus aldehyde. When alcohol is added, it is oxidized to aldehyde, which is a component of the "primary oxidative system" and so a chain reaction is established leading to the complete consumption of all the alcohol added. Instead of ethanol, methanol may be oxidized though to a somewhat lesser degree and, in decreasing order, propanol, isopropanol, isobutanol, and butanol (but not amyl alcohol) are also oxidized, when tested with *d*-amino acid oxidases. When tested with glucose oxidase, only methanol and ethanol are oxidized,

except for traces of propanol and isopropanol. Ethylene glycol and $\beta$-amino ethanol are also readily oxidized, but not glycolic aldehyde, which is the final oxidation product of ethylene glycol. No oxidation occurs with *n*-octanol, benzyl alcohol, glycerin, glucose, lactic acid, malic acid and many others. Although this property of catalase, viz., to induce oxidation of some substrate, can hardly be regarded as merely incidental and must be considered as an essential function, the particular physiological substrate which under natural conditions is exposed to this reaction, is not yet known.

It is remarkable that mammalian erythrocytes, which contain practically no "primary oxidizing systems," are rich in catalase. Here the function of catalase cannot be connected with any primary oxidizing system. It was once thought to have the function of protecting hemoglobin from being oxidized by hydrogen peroxide to methemoglobin (oxyhemoglobin needs no protection, it is not oxidizable). However, when hydrogen peroxide is added, not in bulk, but gradually (as by the action of an oxidative enzyme system, or by means of barium peroxide) it does not protect hemoglobin at all from oxidation to methemoglobin. The apparent "protecting" effect of hydrogen peroxide in large concentration is due to the fact that the oxygen developed by catalase keeps hemoglobin in the oxygenated state, which is not oxidizable to methemoglobin.

That catalase is not an efficient protector from hydrogen peroxide poisoning can be seen also by the fact that catalase-producing bacteria, such as *B. subtilis*, *Proteus vulgaris*, and *Escherichia coli*, are poisoned by very small amounts of hydrogen peroxide, added either directly, or developed on autoxidation of added ascorbic acid. Furthermore, the authors calculate that in tissue respiration the rate of hydrogen peroxide production (assuming this rate to be equivalent to oxygen uptake) is much too high to be completely destroyed by the catalase present in the tissues. In proposing the hypothesis that the function of catalase is the induction of oxidation of a substrate, as yet unknown, the authors warn against considering this as the sole function of catalase, because this hypothesis does not explain why erythrocytes should contain so much catalase.

According to Brandt (28) aqueous emulsions of resting nonautolyzed cultures of *Staphylococcus aureus* contain catalase which

decomposes hydrogen peroxide in a manner characteristic of a unimolecular reaction, at low temperatures. The effect of hydrogen peroxide on the gradual inactivation of this enzyme becomes evident between 30° and 40°, and the temperature dependence of this effect, $Q_{10}$ (for 0 to 20°), is 1.9 or lower. The affinity of the enzyme for hydrogen peroxide is less in emulsions than in the filtrate. In the latter case, the affinity constant is about 0.10. The activity increases from 25° to 65°; thereafter inactivation is rapid. Autolysis liberates inhibiting substances whose effectiveness is diminished by dilution and with progress of the reaction.

The specific protein of peroxidase appears to be able to couple with manganese-hemin, according to spectroscopic evidence. The compound, however, shows no activity as a peroxidase, according to Theorell (29).

It has been claimed by various authors that the complete oxidation of catechol by the enzymatic action of tyrosinase requires only two oxygen atoms per molecule. Wright & Mason (30) show that the amount of oxygen consumed depends upon conditions and may be as high as three oxygen atoms per molecule. With fixed catechol concentration (0.73 mg. per 3 cc.) the final oxygen consumption increased with the amount of the enzyme, up to 2.5 atoms of oxygen. With increase of the enzyme beyond a certain upper limit, there was no further increase in oxygen uptake. On increase of pH, there was an increase in the initial rate of oxygen uptake, but it took more time to complete the reaction. The fact that more than two oxygen atoms were consumed, together with the failure to detect spectroscopically hydroxy-*p*-quinone, does not appear to the authors to be consistent with the mechanism proposed by Wagreich & Nelson in 1935.

Bezssonoff & Leroux (31, 32) demonstrate a "peroxidase activity" of ascorbic acid for oxidation of phenols. For the oxidation of monophenols, copper and iron salts must be present for this effect, but not for oxidation of benzidine or polyphenols. This "peroxidase system" represented by ascorbic acid plus copper differs from ordinary enzymes by the fact that the ascorbic acid-copper system must be present in concentrations of the same order of magnitude as the substrate. Furthermore, according to the composition of the mixture, ascorbic acid may not always serve as a peroxidase, increasing the effect of iron or copper, but under other conditions may behave even as an antioxidant. Thus ascorbic

acid plus copper should not really be considered as a peroxidase in the proper sense.

Ascorbic acid, or cysteine (33), acts as an antioxidant with respect to the oxidation of several polyphenols in alkaline solution.

The peroxidase, active in what is called the "fermentation" of tea, has been further studied (34). The authors find that the oxidation of catechol and of tea tannin is due to the same polyphenol oxidase, in contrast to former results by other authors which did not recognize the inhibitory effect of condensation products of oxidized tannin upon the oxidation of catechol. The enzyme has been purified and found to contain 0.08 per cent copper and 6.60 per cent nitrogen; when purified by adsorption on calcium phosphate it was free from iron. The activity of various preparations was proportional to the copper content. Complete removal of copper inactivated the enzyme but reactivation by addition of copper sulphate was not possible.

The alleged inhibitory effect of sulfhydryl compounds on peroxidase activity is attributed by Randall (35) to the fact that sulfhydryl compounds themselves serve as substrates for peroxidase. The rate of their oxidation by hydrogen peroxide is increased by peroxidase.

Kreke *et al.* (36) found that a compound contained in an alcoholic yeast extract accelerated the decomposition of hydrogen peroxide by catalase. However, Sumner & Sisler (37) show that the alleged activating effect of such an extract is attributable to the protection afforded the catalase in a crude preparation, from its gradual destruction by hydrogen peroxide, and not to an activation of the enzyme.

## Amino Acid Oxidases

Green and associates (38) have further purified the *l*-amino acid oxidase previously described by these authors. Its physical properties have been investigated also by Moore (39). The enzyme was obtained from a crude extract of rat kidney by two successive precipitations with 15 per cent sodium sulphate at pH 5.6, then by at least three stepwise fractionations with ammonium sulphate, with a final yield of at most 1 to 2 per cent. The product was electrophoretically homogeneous but split on ultracentrifugation into two components of equal enzymatic activity. The absorption maximum was approximately 450 m$\mu$ but broader than

that of riboflavin, probably due to an impurity consisting of an iron porphyrin compound. The flavin content was 0.66 per cent. The enzyme was yellow, with greenish fluorescence; the absolute absorption curves was practically superimposable on that of riboflavin. The phosphorus flavin ratio was 1:1. Hence the prosthetic group seems to be a riboflavin phosphate. Since the action on *l*-amino acids runs always parallel to its oxidizing action on lactate, it is suggested that both these reactions are catalyzed by the same enzyme. The two fractions obtained from ultracentrifugation differed only in the degree of molecular aggregation. The molecular weight of the lighter fraction was 120,000, that of the heavier four times as great.

Zeller (40, 41, 42) describes an *l*-amino oxidase in the venom of the snake, *Vipera aspis*, which attacked all *l*-amino acids tested, also diiodotyrosine, dibromotyrosine, and dihydroxyphenylalanine, in short, all *l*-amino acids with unsubstituted amino groups, but not the *d*-forms, nor thyroxin or glycine. In contrast to the mammalian enzyme it does not oxidize *l*-proline or *l*-hydroxyproline. The reaction rate increases with the chain length. There is no catalase in snake venom, and the reaction follows the scheme:

$$\text{First step: amino acid} + O_2 \rightarrow \text{keto acid} + NH_3 + H_2O_2$$

$$\text{Second step: keto acid} + H_2O_2 \rightarrow RCOOH + CO_2 + H_2O.$$

After addition of catalase the reaction is

$$\text{amino acid} + \tfrac{1}{2}O_2 \rightarrow R \cdot CO \cdot COOH + NH_3.$$

A second amino group prevents oxidation. Glycine and ornithine are not oxidized. Also a second carboxyl group prevents oxidation. Aspartic and glutamic acids are not oxidized, but asparagine and glutamine are, as is also glutathione.

*Neurospora* (43) contains a *d*-amino acid oxidase similar to the mammalian one with an optimum pH of 8.0 to 8.5 in pyrophosphate buffer.

Epps (44) finds that the tissue concentration of human amino acid oxidase is a function of age (the concentration in the mucosa of the ileum shows no such dependence however). The kidney is relatively deficient in the enzyme prior to the third month. Many children show no amino acid oxidase activity in the duodenum during the first month. There is no significant gradation with age

in the amino acid oxidase activity in the colon.

Neilands (45) describes a trimethylamino oxidase, $N(CH_3)_3 \rightarrow NO(CH_3)_3$, in the muscle of the cod fish, which is inhibited by cysteine but not by thioglycolate or carbon monoxide. The development of $N(CH_3)_3$ is due to bacterial growth. Its formation is inhibited by sodium azide or sodium cyanide. He thinks that cyanide stabilizes one fraction of the enzyme, a metallo group, which allows the remainder of the enzyme to become saturated with the substrate. Reduction of $NO(CH_3)_3$ appears to require an oxidizable substrate, a specific dehydrogenase and one or more carriers.

The oxidative desamination (46) of adenine to hypoxanthine, or of guanine to xanthine, or the two corresponding reactions involving adenosine or guanosine instead of adenine or guanine, is due to four different enzymes. In invertebrates only the two enzymes acting on the purines themselves are found. Desaminases for nucleosides are a later acquisition in phylogeny. The salamander is exceptional, having no guanosine oxidase.

The extensive studies by Hellerman and associates (47) on enzyme inhibition belong to a chapter on the mechanism of enzymatic activity. Here little of their extensive experimental and theoretical material can be mentioned. The authors have studied the inhibitory effect of quinine, atabrine, and related compounds on the activity of *d*-amino acid oxidase. These substances inhibit strongly at low concentrations of flavin-adenine-dinucleotide, but only slightly at high concentrations, which suggests their competition with the nucleotide for the specific protein, and not with the substrate, the amino acid. In the case of atabrine this conclusion is not strictly applicable.

The nature of the enzyme capable of oxidative decarboxylation of oxaloacetate to malonate and carbon dioxide, described in 1944, has been clarified (48). The enzyme has been identified with metmyoglobin which can be obtained in a pure, crystalline state, from fresh, dialyzed, water extracts of horse heart muscle (reported by several authors from 1932 to 1936). Hemoglobin and cytochrome-*c* show no activity. There is an initial lag period in the consumption of oxygen which can be reduced by small amounts of hydrogen peroxide, and increased by catalase. During the oxidation, the metmyoglobin is converted into a green pigment, then to a light brown pigment with no sharp absorption bands, and which is

not active as an enzyme. The oxidation, plotted against time, always shows an S-shaped curve and, when the amount of the enzyme is limited, does not go to completion, the degree of incompleteness depending on pH, concentration of buffer, and the reacting reagents.

A desaminase which catalyzes the reaction, adenosine→inosine, is contained in "Takadiastase" (49). In phosphate buffer it has a broad maximum of activity from pH 5 to 8; in succinate buffer the maximum is rather sharp at pH 6.2. The discrepancy is explained by assuming an influence upon the dissociability of the enzyme-substrate compound depending on the buffer used.

## Flavin Enzymes

To the flavin proteins referred to in the section on amino acid oxidases several others will be added in this section. One of the crystalline proteins obtained by Kunitz (84) as a by-product during the preparation of hexokinase from bakers' yeast is a yellow protein which has been studied by Ball (50). It has a prosthetic group which is obviously riboflavin, according to its absorption spectrum and also because it gives rise to a typical lumiflavin on irradiation with ultraviolet light. However, the absorption spectrum in the ultraviolet indicates that it contains, in addition, another prosthetic group of unknown nature. The present knowledge of the flavin enzymes may be summarized, according to Ball, as follows: Two flavin prosthetic groups had been known for some time; riboflavin phosphate, in Warburg's original yellow respiration enzyme, and flavin-adenine-dinucleotide, the coenzyme of *d*-amino oxidase. Now one has to add two more, "atypical," flavoproteins, the one just described, and another described by Green *et al.* (51) which is of a more brown color and contains a flavin-adenine-dinucleotide. Its prosthetic group is not released on denaturation of its protein carrier. It also is of unknown catalytic function. Furthermore, among enzymes of known catalytic action, xanthine oxidase (52), liver aldehyde oxidase (53), and glucose oxidase from *Penicillium notatum* (54, 55) are flavoproteins.

Kelsey *et al.* (56) found that minced rabbit liver acts upon quinine, and the reaction product isolated was identified by Mead & Koepfli (57) as carbostyril, quinine being oxidized with replacement of one hydrogen atom in the quinoline ring by a hy-

droxyl group. The enzyme responsible for this oxidation has been purified by Knox (58) to the extent of 5 per cent purity. It contains riboflavin. The flavin is reduced anaerobically by cinchonidin. As a result of aerobic oxidation hydrogen peroxide is formed. The optimum pH is 7.4 to 7.6. It reacts with oxygen directly but more rapidly with the intermediation of methylene blue. The enzyme is rapidly destroyed during the catalysis. Quinoline, isoquinoline, and some pyridine derivatives are oxidized by the enzyme. Among physiologically important compounds, N-methylnicotinamide is most rapidly oxidized. The enzyme has properties similar to the liver aldehyde oxidase, a flavoprotein, and is associated with it. The simultaneous oxidation of aldehyde and of quinoline is slower than their separate rates. Hence, the enzyme concerned with these two reactions appears to be the same, and is considered as a flavoprotein endowed with two functions. Rabbit liver appears to be the only satisfactory source of the enzyme. It can be extracted with water, is resistant to heating for five minutes to 60°C. and is precipitated at between 25 and 40 per cent saturation with ammonium sulfate. Successive precipitations yield a yellow-red solution which remains active in ammoniacal ammonium sulfate over a month.

### Other Dehydrogenases and Oxidases

According to Bergstermann & Stein (59) quinone is a powerful inhibitor for succinic dehydrogenases, presumably because it blocks sulfhydryl groups of the enzyme. The inhibitory effect upon the enzyme is even greater in the absence of the substrate, succinate. The protective action of the substrate is still greater in preventing the destructive effect of tellurite, selenite, or arsenite.

Ribonuclease inhibits succinic dehydrogenase according to Zittle (60). Also mononucleotides exert such an inhibition. However, ribonuclease inhibits even when the concentration of the mononucleotides arising from its action is too low to account for the inhibition. Furthermore, the inhibitory effect of mononucleotides is not directed toward the cytochrome part of the system, whereas ribonuclease inhibits both cytochrome oxidase and succinic dehydrogenase. Schneider (61) confirms these experimental results but finds that the effect on succinic dehydrogenase is due to an impurity in the ribonuclease preparation.

The activity of succinic acid dehydrogenase from certain endo-

crine glands and other tissues was tested by McShan *et al.* (62). Adrenal gland had half the activity of liver and twice that of pituitary, uterus, thyroid, thymus, and testis. Little activity was found in pancreas, prostate and least of all in seminal vesicle.

Werkman and associates (63) prepared a cell-free enzyme preparation of oxidative activity from *Micrococcus lysodeikticus*. In such cell-free preparations, problems with respect to permeability are circumvented. The cells are lysed by saliva or egg white (lysozyme), or treated with acetone. Such preparations are not capable of oxidizing all substrates such as succinate or acetate, but they do oxidize acetyl phosphate. This reaction is greatly enhanced by sodium chloride which probably inactivates the lysozyme which otherwise may cause the lysing process to proceed too far and finally may lead to gel formation and then render the enzyme inactive. Preparations, thus kept in an active state by sodium chloride are able to metabolize acetyl phosphate and to transfer the phosphoric group to adenylic acid. A comparison of the effect on acetate and acetyl phosphate suggests a path for the oxidation of acetyl phosphate not leading through the stage of free acetate as intermediary.

Herrmann & Boss (64) obtained tissue preparations from the ciliary body which accelerated the aerobic oxidation of dihydroxyphenylalanine in the absence of cytochrome-*c*, but not that of hydroquinone or catechol which suggests a specific "dopa" oxidase, the existence of which had been postulated by Hogeboom & Adams in 1942 from experiments with melanotic tumors. The isolated pigment granules, allegedly an inert end product or deposit, contain "dopa" oxidase, cytochrome oxidase, and succinic dehydrogenase.

Diaphorase, a flavin-adenosine-dinucleotide, which catalyzes the oxidation of reduced coenzyme I (which does not react with oxygen directly), stimulates growth in tissue cultures of fibroblasts (65). Creatine has a similar effect.

Flexner & Flexner (66) have studied succinic dehydrogenase during development of the cerebral cortex in the fetal pig by measuring either the oxygen uptake in the presence of cytochrome-*c* plus cytochrome oxidase, or the reduction of ferricyanide in the absence of the cytochrome system. In the earlier stage they found only 35 per cent of the activity present in the adult. Increase of activity began between sixty-eight to seventy-five days of gesta-

tion and from then was equal to that of the tissue at birth.

Hogeboom (67) in a preliminary note describes the preparation of a soluble, clear succinic dehydrogenase from the "large granulae" or "mitochondrial fraction" according to Claude, from rat liver, by extraction of the acetone dried material with 0.01 M $NaHCO_3$. [Compare also with references (25, 26).]

Green and his group have found occasionally that *l*-amino acid oxidase preparations are able to oxidize also *l*-hydroxy acids, the two properties running always parallel during purification of the enzyme (68). The specific lactic and malic dehydrogenases can be distinguished from the enzymes investigated here in two respects: they are specific for just one hydroxy acid, and they require diphosphopyridine nucleotide, whereas the others act on a variety of hydroxy acids and without the nucleotide; furthermore they contain a flavin phosphate prosthetic group. The *d*-amino acid oxidase of animal tissues does not show a comparable effect on *d*-hydroxyamino acids. The action of the enzyme was tested manometrically in the presence of lactate and catalase by measuring oxygen uptake. The rate depends largely on pH, the optimum being pH 8, and no activity occurring below pH 6 or above pH 10. Among fourteen *l*-hydroxyamino acids tested, only $\alpha$-hydroxy isobutyric acid was not oxidized by this enzyme. The presence of a second carboxyl group, which renders the substrate inactive in the *l*-amino acid series, had a similar effect also in the *l*-hydroxyamino acid series. Thus aspartic acid was quite inert and malic acid was but slowly oxidized. Glycolic acid was oxidized, showing that an optically inactive substrate is accessible to the enzyme. The reaction products, in the presence of catalase and the enzyme, are one molecule of keto acid from one molecule of hydroxy acid, plus one atom of oxygen. In the absence of catalase the stoichiometry is difficult to interpret for obvious reasons. The *l*-amino acid oxidase was resolved in the ultracentrifuge into two fractions of equal activity, of M.W. 138,000 and 550,000, respectively. The activity of these two fractions against *l*-hydroxy acids was also the same, another evidence for the identity of the enzyme, which acts either on amino or on hydroxy compounds.

The catalytic oxidation of ascorbic acid may be effected by various enzymatic systems. There is a specific ascorbic oxidase described by Diemair & Zerban (69), but oxidation can also be achieved by intermediation of the system catechol-oxidase plus

catechol (Keilin & Mann). Bertrand (70) shows that in most enzymatic systems of vegetable origin neither of these pathways exists, but that usually laccase (Bertrand, 1894) performs this oxidation. The specificity of laccase is much less than that of these other enzymes. It acts directly on *o*- and *p*-diphenols and diamines, also on benzidine, and on ascorbic acid. Thus in systems such as, substrate→dehydrogenase→ascorbic acid→"oxidase"→oxygen, the "oxidase" is most frequently represented by laccase, and not by any one of the more specific oxidases.

Valdiguie *et al.* (71) describe an enzyme called carbinol dehydrase, found in tissues of higher plants, which oxidizes isopropyl alcohol to acetone both aerobically and, in the presence of methylene blue, 2,6-dichlorophenol-indophenol, quinone, or alloxan, also anaerobically. A possible intervention of sulfhydryl compounds, lactoflavin or nicotinamide in this reaction has not yet been cleared up.

Mushroom tyrosinase (72) is able to catalyze the oxidation, by oxygen, of tyrosine within the protein molecule without hydrolysis being necessary. The oxidation was studied by several different methods. The following proteins were tested: trypsin, pepsin, chymotrypsin, casein, peptone, insulin, and hemoglobin. No change was observed in the tyrosine-free protein gelatin, or in gramicidin. Certain tyrosine-containing proteins were resistant to the enzyme, such as egg albumin, serum albumin, tobacco mosaic virus, human $\gamma$-globulin, and bovine fibrinogen. After digestion with trypsin, the tyrosine of those proteins became oxidizable. The oxidation of the tyrosine group had no effect on the activity of pepsin, trypsin or chymotrypsin, probably because only a small fraction of the tyrosine groups was oxidized and the oxidation of each tyrosine group did not go very far.

## Decarboxylation Reactions, Simple and Oxidative, and Their Reversal

The enzyme, *l*-arginine decarboxylase, is characterized by Taylor & Gale (73, 74) as follows: pH optimum 5.25, affinity constant 0.00075. For *l*-ornithine decarboxylase the corresponding values are 4.25, and 0.027. The arginine decarboxylase may be resolved into an apoenzyme and a coenzyme, pyridoxine phosphate, by precipitation with ammoniacal ammonium sulphate. The ornithine enzyme resolves into these components spontaneously. The

glutamic acid enzyme has not yet been resolved. Those enzymes resolvable are more sensitive with respect to inhibition by iron and hydrazine than those that are not resolvable.

The *l*-cysteic acid decarboxylase found in some mammals by Blaschko (75) (in kidney of rats, but not of dog, cat or man) is inactivated by dialysis. A water soluble, heat labile factor is removed. The enzyme acts quite specifically. Homocysteic acid seems to have an affinity for the enzyme, manifested in inhibition of its action upon cysteic acid, but itself is not affected. The activity of the enzyme *in vitro* is not increased by addition of thiamine or cocarboxylase.

Jackbeans contain a potent carboxylase requiring cocarboxylase and magnesium (76). Soybeans contain approximately as much thiamine as jackbeans, but no carboxylase. The use of jackbean meal extract for manometric estimation of urea may lead to erroneous results in the presence of pyruvic or oxaloacetic acid.

Glutamic acid decarboxylase has been found in higher plants such as squash, avocado, and green peppers (77). The enzyme can be obtained in the form of a clear solution and can be resolved by dialysis at pH 6.4 into a protein carrier and a prosthetic group. Since addition of pyridoxal and especially of a synthetically prepared pyridoxal phosphate restores the inactive apoenzyme, the latter may in all probability be considered as the prosthetic group.

Glutamic acid decarboxylase of carrots, according to Schales & Schales (79), has its highest activity at pH 5.3 to 5.9 and no activity below 4.0 and above 7.5. The time-activity curve is not that of a monomolecular reaction, but becomes so on addition of pyridoxal phosphate. The affinity constant is approximately $3.6 \times 10^{-3}$ at pH 5.7 to 6. Hydroxylamine strongly inhibits the initial velocity of the reaction.

Takadiastase is a good source for a desamidizing enzyme (78), converting adenosine to inosine. In phosphate buffer there is a broad activity maximum from pH 5 to 8, in succinate buffer a sharp maximum at pH 6.2.

Utter & Wood (80) have continued their studies of carbon dioxide fixation. As reported in last year's Review (81), the exchange of the isotopic carbon ($C^{13}$) between oxaloacetate and labeled bicarbonate takes place in the presence of adenosinetriphosphate (ATP). The general importance of this reaction is

not yet established, since carbon dioxide fixation has been found also to occur in oxalosuccinate by Ochoa, and in pyruvate by Lipmann & Tuttle, and Utter, Lipmann & Werkman (cf. 81). In the present studies (80) pigeon liver extracts were used as enzyme source. The determination of oxaloacetate depends on measurement of the carbon dioxide obtained from it on boiling, and is not entirely specific. For this reason the source of the labeled carbon dioxide obtained may also be such compounds as acetoacetate or oxalosuccinate. However, the authors eliminate the presence or the formation of such compounds in their experiments. They conclude that the only reaction concerned with carbon dioxide fixation in their experiments is the reversal of the oxidative decarboxylation of oxaloacetate. The authors propose an explanation for the poor correlation between carbon dioxide fixation and decarboxylation of oxaloacetate as expected from the reversibility of the process.

Specific decarboxylase preparations for *l*-lysine, for *l*-tyrosine, for *l*-histidine, for *l*-glutamic acid, and for *l*-ornithine are used by Gale (82) for the quantitative analysis of protein hydrolysates.

## Phosphorylation

The first step in the oxidative metabolism of glucose is its phosphorylation. Hexokinase, the enzyme catalyzing the formation of glucose-6-phosphate, by transfer of the phosphate group from ATP to glucose, has been obtained in a pure and crystalline state simultaneously and independently by Cori (83) and by Kunitz (84) and their associates.

The methods used by these authors were similar and started from the original procedure of Meyerhof, i.e., by plasmolysis of bakers' yeast with toluene, fractional precipitation with alcohol at low temperature, and by taking advantage of the fact that the stability of the enzyme is remarkably increased in the presence of its substrate, glucose. Cori used, in addition, adsorption on aluminum hydroxide and elution with phosphate buffer, whereas Kunitz used only fractionation with ammonium sulphate and alcohol, during which procedure he was able to prepare, in the crystalline state, three more new proteins, one of which is yellow, and which is discussed in another section of this review. Substrates for these additional proteins have not yet been found. After removal of these "inert" protein fractions, hexokinase was obtained by

crystallization from ammonium sulphate and repeated recrystallization. Its purity was tested by the solubility test, by electrophoresis, and by ultracentrifugation at pH 5.6. At pH 6.0 a double boundary was established, probably due to the influence of centrifugation. According to Kunitz the protection of the enzyme exerted by glucose can also be obtained with the following sugars, in descending order: glucose, mannose, sucrose, fructose; galactose has little effect, maltose none. This order is not the same as that in which the phosphoric group is enzymatically transferred from ATP. Here the order is: glucose, fructose, mannose; no reaction with galactose, maltose, or sucrose. The isoelectric point, determined by coating collodion particles with the protein, is at pH 4.5 to 4.8. From diffusion and sedimentation data the molecular weight is calculated to be 96,600. Magnesium is requisite for its activity. According to Cori *et al.* the protective action of glucose is correlated with the inhibition of proteolytic enzymes in the yeast extract.

## Oxidation of Fatty Acids

According to Györgyi & Tomarelli (85), butter yellow (N, N-dimethyl amino-azobenzene) retards the autoxidation of linoleic acid. It acts synergistically in enhancing the antioxidative property of rice bran extract or hydroquinone, but not that of $\alpha$-tocopherol. Out of a number of antioxidants tested, only $\alpha$-tocopherol exerted any appreciable inhibition of the catalyzed oxidation of linoleic acid, or of carotene, due to the soybean lipoxidase. Diphenylamine, with the enzymatic system, displayed a slight action, while hydroquinone, its methyl and benzyl esters, diphenyl esters, or butter yellow, showed no inhibition of the antioxidant effect.

The oxidation of unsaturated fatty acids, either spontaneous autoxidation or enzymatically induced oxidation, has been studied spectrophotometrically by Bergström (86). The method is based on the fact that on oxidation a chromophoric group is developed which can be accounted for by the development of peroxide compounds. The rate of development of the characteristic band is, under certain conditions, parallel to the rate of oxygen consumption.

Lehninger (87), in continuation of his studies on oxidation of saturated fatty acids, studied the mode of activation of the

enzyme contained in a rat liver suspension. The presence of adenylic acid, cytochrome-*c*, magnesium, and inorganic phosphate is requisite for the oxidation to acetoacetic acid which occurs only together with the oxidation of some other oxidizable metabolite. The oxidation can be coupled to a specific one-step oxidation, that of $\alpha$-ketoglutaric acid to succinate. However, in the presence of ATP, the oxidation of fatty acids is entirely independent of such co-oxidation. It can be concluded therefore that the co-oxidation has the role of providing ATP. Only ATP, not ADP nor any other phosphate ester, is effective. The activated oxidation is sensitive to sulfhydryl reagents. Only the fatty acids, not their esters (unless enzymatically hydrolyzed) are oxidized. The author suggests that the activation by ATP depends on a phosphorylation of the fatty acid. In another paper (88) the synthesis of such phosphate esters of some higher fatty acids is described; Lipman's method for the synthesis of acetylphosphate is essentially followed. The esters obtained are more hydrophilic than the fatty acids themselves, relatively unstable in water, but more stable than acetylphosphate, and are rapidly hydrolyzed within the tissues.

In a subsequent paper (89) the author shows that the oxidation of caprylate by the rat liver enzyme in presence of ATP, magnesium, and malonate goes completely to acetoacetate:

$$C_7H_{15}COOH + 3O_2 \rightarrow 2CH_3COCH_2COOH + 2H_2O.$$

The same enzyme, in the presence of ATP, magnesium, and malonate and in the absence of oxaloacetate, oxidizes pyruvate quantitatively to acetoacetate:

$$2CH_3CO \cdot COOH + O_2 \rightarrow CH_3COCH_2COOH + 2CO_2 + H_2O$$

However, in the presence of oxaloacetate the yield of this reaction diminishes and extra citrate accumulates (Krebs condensation). Furthermore, when fatty acids are oxidized by this enzyme in the presence of fumarate and malonate, the yield of acetoacetate is diminished, and extra citrate, $\alpha$-ketoglutarate and succinate accumulate in such amounts as to account quantitatively for the carbon of fatty acids diverted from acetoacetate production. Fatty acids of the $C_{2n-1}$ series are oxidized and form acetoacetate and citrate at approximately the same rate as their $C_{2n}$ neighbors.

The same author (90), after having studied the conditions

under which a saturated fatty acid is oxidized in liver homogenate, investigated the problem for heart muscle homogenate. The latter is capable of oxidizing caprylate in the presence of adenosine nucleotide and on simultaneous oxidation of fumarate. The end-product is succinic acid, provided succinic dehydrogenase is inactivated by malonate. Succinate is accumulated quantitatively corresponding with the assumption that a two-carbon fragment from a fatty acid combines with oxaloacetate to form tricarboxylic acids and ultimately succinate. Citrate or acetoacetate does not accumulate. Acetoacetate is readily oxidized with formation of succinate. The result strongly suggests that oxidation both of fatty acid and of acetoacetate proceeds through the Krebs tricarboxylic acid cycle in heart muscle homogenate.

Experiments (91) with slices or homogenates of rat kidney with labeled sodium acetate, containing the isotope $C^{13}$, showed that about 50 per cent of the acetate was converted to ketone bodies, whereas in rat heart muscle no intermediate ketone bodies could be found during the oxidation.

## Oxidation of Cyclic Compounds

The enzymatic oxidation of uric acid, according to Klemperer (92), produces only a fraction of the carbon dioxide expected on complete oxidation to allantoin. Other products of the oxidation are uroxamic acid and hydroxyacetyl-diureine-carboxylic acid:

```
       H
OC—N—C(COOH)—NH
 |      |          |
HN——C(OH)—N—CO
                  H
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In the oxidation and detoxication of cyclic compounds, ascorbic acid is involved, according to Ekman (116). After ascorbic acid, excretion of polyphenols increases, but that of urochrome A decreases. The author ascribes this function of ascorbic acid to the hydrogen peroxide developed on its oxidation. The primary oxidation products of benzene are polyphenols which in their turn are further oxidized by hydrogen peroxide and peroxidases, without the intervention of ascorbic acid. Ascorbic acid thus acts as a detoxifier, but its action is inhibited by excessive amounts of ascorbic acid, because the further oxidation of the polyphenols is inhibited. In this case urochrome A is also diminished.

## Sulfhydryl Compounds

The activity of cytochrome oxidase, succinoxidase, succinic-, lactic-, and glucose-dehydrogenase, and of catalase is depressed by phenylmercuric nitrate (93); an inhibitory effect of *p*-chloromercuribenzoate on oxidase and catalase had not been observed by Barron & Singer (117). Cook *et al.*, for this reason, think it probable that a sulfhydryl group is essential for these enzymes. The depression of respiration of yeast brought about by phenylmercuric nitrate (94) is prevented by cysteine and homocysteine, but not by cystine or methionine, which also suggests an essential sulfhydryl group in the enzymes involved with respiration, although the effect of the drug may not be due to reaction with sulfhydryl groups alone.

The rate of enzymatic oxidation of glutathione (95) is increased by coenzyme I in the absence of cytochrome-*c*, and also the induction period is decreased. The enzyme used was that contained in a cell-free homogenate of mouse kidney. ATP has no effect. Ascorbic acid stimulates the catalytic oxidation of glutathione both in the absence and in the presence of cytochrome-*c*.

The antithyroid action of some thiol compounds cannot be explained by their antiperoxidase activity (96) as Dempsey suggested in 1944. Thiol compounds do not inhibit peroxidase but are reducing agents reacting on the substrate of peroxidase.

## Miscellaneous Subjects

The inhibitory effect of aromatic diamines (such as arise from metabolic reduction of the carcinogenetic dye "butter yellow") upon certain enzymes, urease and succinoxidase, was correlated with the formation of their oxidation products, free radicals of the type of Wurster's dyes, by Kensler, Dexter & Rhoads in 1942. Elson & Hoch-Ligeti (97) agree that oxidation products are responsible for the effect, but they are supposed to be of quinonoid structure. The reviewer cannot see much difference in the point of view. The oxidizability depends on the ease with which a relatively stable semiquinone radical can be formed (it can, e.g., in *p*-phenylene diamine, but not in aniline) and the oxidation has to proceed via such a free radical. Whether it is this free radical itself which displays this effect, or some further oxidation product, is difficult to decide. Anyhow, this action of the amines is correlated with the ease of oxidation, which in its turn is correlated with the ease of formation of a relatively stable free radical as a first step.

In studies on some wood-destroying fungi (98), dehydrogenating enzymes were investigated. In these studies the authors propose resazurin as an indicator for a two-step reduction, each step being a bivalent oxidation without establishment of any appreciable amount of intermediary free radical. This blue dyestuff, on reduction, first loses irreversibly one oxygen atom, becoming resorufin, of pink color, and on further reversible reduction, becomes colorless dihydroresorufin. Once the reduction has taken place, reoxidation by later exposure to air can go only to the pink stage; thus the occurrence of reduction can be recognized by this indicator even when later the system is exposed to a reoxidizing agent.

Terroine (99) investigated the influence of dyestuffs of the nature of redox indicators, on the metabolism of rats, starting from the idea that incorporation of large amounts of hydrogen acceptors, should modify the metabolism. Such an influence was found with regard to protein metabolism, but not in respect to that of purines, or of creatine. The influence on protein metabolism depended on the normal potential of the dye. Dyes with a potential from $-0.115$ to $-0.03$ volts (toluylene blue, thionine, cresylblue) decreased the excretion of nitrogen, owing to a lowering of protein oxidation. On the other hand, with dyes with potentials more negative than $-0.34$ volt (Nile blue, phenosafranin, Janus green, neutral red), nitrogen excretion was increased. Methylene blue is the transitional member of these two groups.

The rate of decoloration of toluylene blue (100) by ascorbic acid was proportional to the concentration of the latter. The rate of decoloration by cysteine increased much faster than the concentration.

Potter (101) describes experiments to show that the energy of respiration can be stored as high energy phosphate bonds, even in cell-free tissue homogenate. He uses systems in which the energy of oxidation is stored up finally as creatine phosphate by the intermediation of ATP. Proper choice of the tissue is necessary. An essential factor seems to be that the homogenate, prepared at 0°C., be immediately diluted at 37°C. to such an extent that autolytic processes will not interfere.

The effect of the rodenticide, $\alpha$-naphthylthiourea, or "ANTU," seems to be correlated with its effect upon certain oxidations (102). Dogs and rats poisoned with the drug show marked hyperglycemia

and depletion of liver glycogen. The compound inhibits potato tyrosinase; its efficiency in this respect and that of some other compounds can be expressed by the concentration at which the inhibition amounts to 50 per cent: "ANTU" at $8 \times 10^{-6}$ *M;* phenylthiourea, $2.5 \times 10^{-7}$ *M;* allyl thiourea, $9 \times 10^{-1}$ *M;* and thiourea, $1 \times 10^{-3}$ *M*. The inhibition can be prevented by iodine or cupric salts, but the inhibition, once established, cannot be reversed by them. The thiourea compounds also inhibit oxidation of ascorbic acid catalyzed by copper, but not the oxidation of cysteine. There is no effect on cytochrome oxidase or succinic dehydrogenase.

An interesting and still puzzling problem has arisen from Granick's (103) studies on the metabolism of ferritin. There is a cyclic conversion between the ferrous and the ferric state involved in the iron metabolism. Iron is absorbed in the intestine in the ferrous state and stored in the intestinal wall as ferritin in the ferric state. The ferrous iron, in so far as it is absorbed (and not stored), is converted in the blood stream immediately to the ferric state, the iron being combined with some of the serum proteins, but not with ferritin. This fraction has been shown by Schade (104) to be a subfraction of serum globulin. The excess iron is deposited in the ferric state in the form of ferritin in the liver, spleen, and bone marrow. When ferritin is being used for the formation of hemoglobin, reduction of the ferric to the ferrous state is postulated.

*Oxygen-carrying compounds.*[2]—Compounds capable of reversibly combining with, or releasing, oxygen according to the oxygen pressure, are represented in nature by hemoglobin and hemocyanin. In hemoglobin the ratio is 1 Fe:1 $O_2$; in hemocyanin 2 Cu:1 $O_2$. There has been, until recently, no other compound either occuring in nature, or prepared in the laboratory known to exhibit this important property. A successful attack on the problem of the nature of reversible oxygenation has now been made to such an extent that the final solution seems to be only a matter of time. Utilizing some isolated observations by Pfeiffer (105) and by Tsumaki (106), Calvin (107 to 111) and associates describe metal complex compounds, especially of cobalt, which can combine reversibly with oxygen. Only a few of the fundamental facts may be recorded here. On mixing (*a*) a cobaltous salt, (*b*) an aldehyde or ketone, usually a salicyl-aldehyde derivative or an *o*-hydroxyacetophenone derivative, and (*c*) an amine component, one can obtain two types of chelate cobalt complex compounds each of which usually exists in sev-

[2] The following sections were received too late for inclusion in the body of the text. Editor.

eral modifications as to crystal form and other properties, one of them being capable of combining reversibly with oxygen. Oxygen is absorbed either by the solid compound or by its solution in an adequate organic solvent, and is released on heating, or on lowering the oxygen pressure. The reversibility is usually not quite perfect. After several cycles a progressive irreversible change takes place.

Compounds of two types appear to be formed which differ in the cobalt:oxygen ratio. Those of type I are paramagnetic, exhibiting spin of one unpaired electron, and after combination with oxygen, are diamagnetic. Compounds of type II are paramagnetic, with three unpaired electrons, and, after absorbing oxygen, are paramagnetic with one unpaired electron. The speed of absorption of oxygen varies with the particular compound. For each compound it always increases with increasing temperature, but the equilibrium, at a fixed oxygen pressure, is displaced in favor of the oxygenated compound by decreasing the temperature. The oxygen saturation curves resemble those of hemoglobin in so far as they are not representative of a simple equilibrium between one chelate molecule and one oxygen molecule.

A further advance in the studies of oxygen carriers has been published, so far only in a preliminary note, by Burk *et al.* (112). These authors observed an inhibitory effect of cobalt on the growth and respiration of various microorganisms and animal tissues, especially tumors (113, 114). This inhibition is reversibly overcome by histidine. Guided by this observation, the authors studied the cobalt compounds of histidine and found that the nearly colorless complex of the ratio $Co^{++}$:histidine = 1:2, absorbs one molecule of oxygen for two cobalt atoms, turning brown in a reversible manner, oxygen being released and color discharged either on decreasing the oxygen pressure or by raising the temperature. The speed of oxygenation is largely increased with increase of temperature, but the equilibrium is displaced in favor of oxygen absorption with decreasing temperature. The oxygenated complex reacts at a much lower speed, with another molecule of oxygen in an irreversible manner, forming a pink complex. The authors think that neither the first reversible uptake of oxygen, nor the second, irreversible one, changes the cobaltous state to the cobaltic. They so concluded from the observation that no hydroxyl ions were produced on oxygenation. The physiologically interesting property of this complex is that oxygenation takes place in aqueous solution. The reason that histidine, specifically, is endowed with this property, is supposed to be that not only is the amino group attached to cobalt, but also the imino group, thus forming a chelate compound. The reviewer, in a preliminary experiment, found this complex, when nonoxygenated, paramagnetic but diamagnetic when oxygenated.

*Porphyrin compounds as growth factors.—Hemophilus influenzae* requires iron-porphyrin compounds for respiration and growth, just as other aerobic cells or microorganisms, but it is unable to synthesize these compounds. For this reason, addition of various porphyrin compounds to the nutrient medium reveals their function in this microorganism, as Granick & Gilder (115) have shown. Added protoporphyrin IX promotes growth: the bacillus is able to insert iron so that iron-protoporphyrin can be used as the prosthetic group of the respiratory enzyme. No other porphyrin (hemato-, meso-, deutero-, or copro-) can be used instead, at least in the smooth strains of the bacillus. When, however, the iron compound of any of these porphyrins is added instead of the free porphyrin, growth is

promoted. Hence, this microorganism can use any iron-porphyrin as growth factor, but it can insert iron only into protoporphyrin. However, although growth is promoted by any iron-porphyrin, the faculty of reducing nitrate to nitrite is established only by the iron-protoporphyrin. The iron-free porphyrins other than protoporphyrin, when added together with protoporphyrin, inhibit the growth-promoting effect of protoporphyrin; the inhibition being complete with a tenfold excess of the inhibitor. These porphyrins compete with iron-protoporphyrin for combination with a specific protein, the apoenzyme. There is formed what may be called a pseudo-respiratory enzyme which is inactive due to lack of iron. None of the porphyrin compounds can be utilized when the propionic acid groups are esterified. The specific function of protoporphyrin is attributed to its vinyl groups. One rough strain of this organism, the "Turner" strain, can insert iron also into mesoporphyrin provided it is present in low concentration. In high concentration, mesoporphyrin inhibits growth as it does in the smooth strain.

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# PROTEOLYTIC ENZYMES[1]

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## Nomenclature

The rapidly increasing number of known enzymes which hydrolyze peptide linkages has stimulated efforts to classify these enzymes and to develop a rational nomenclature. In general, the proposal offered several years ago (1) to separate the proteolytic enzymes into two broad groups, the exopeptidases and the endopeptidases, has been adopted widely. This separation is based upon the difference in specificity with respect to the requirement, in the "backbone" of the substrate, of a free alpha-amino or alpha-carboxyl group adjacent to the sensitive peptide linkage. The exopeptidases require such groups and therefore are restricted in their action to terminal peptide bonds, while the endopeptidases, which do not require free terminal amino or carboxyl groups, are capable of splitting central peptide bonds of proteins and suitably substituted peptides.

The problem of classifying the exopeptidases has not aroused controversy since it has long been the custom to speak of "aminopeptidases" and "carboxypeptidases," thus describing the enzyme specificity with respect to the nature of the requisite terminal group of the appropriate substrate. The recognition of side-chain specificity among the aminopeptidases has led to the introduction of terms such as "leucine aminopeptidase" (2). Further studies may well establish the existence of aminopeptidases with other side-chain specificities, and these could be designated "tyrosine aminopeptidase," "arginine aminopeptidase," etc. Thus far, a separation of the known carboxypeptidases according to side-chain specificity has not been necessary.

In the case of the endopeptidases, however, the situation is more complex. Designation of a proteinase as an endopeptidase, and reference to its side-chain specificity would not, in itself, be sufficient to differentiate the known proteinases. Thus, the term "tyrosine endopeptidase" could apply equally to pepsin and to

[1] This review covers the period from December 1, 1945 to December 1, 1946.

chymotrypsin. More complex terminology might perhaps be devised but it is doubtful whether well-established simple names such as "pepsin," "trypsin," etc. would readily give way to an involved polysyllabic classification. The finding that several of the known endopeptidases could be grouped together on the basis of a common backbone and side-chain specificity led to the suggestion (2) that the members of each "homospecific" group be designated with reference to the best-known member of that group. Thus, the endopeptidases homospecific with crystalline pancreatic trypsin were termed "trypsinases," while those related to crystalline swine pepsin were termed "pepsinases." In a sense, this terminology violates the conventions for the nomenclature of enzymes, since the suffix "ase" is customarily added to the root derived from the name of the substrate. Perhaps the terms "homotrypsins," "homopepsins," etc., would be less open to objection on this score.

Recently, the idea has been revived that the proteolytic enzymes be classified on the basis of their pH optima, and the terms "acidoproteinase," "neutroproteinase," and "basoproteinase" have been offered for consideration (3, 4). Such a classification has disadvantages in that it obscures the specificity characteristics of the various known proteinases and separates enzymes with identical specificity into different categories. Furthermore, there is good evidence that the pH optima of the proteinases vary according to the chemical nature of the substrate, thus necessitating the inclusion of the same enzyme in two categories.

## Exopeptidases

*Aminopeptidases.*—It has long been recognized that certain of the aminopeptidases are markedly activated upon the addition of metal ions, in particular manganese, magnesium, zinc, and cobalt (5). In a recent study (6), it has been shown that the activation, by manganese ions, of purified leucine aminopeptidase from swine intestinal mucosa involves the formation of a dissociable manganese-protein complex. With *l*-leucinamide as the substrate, data were obtained which were in accord with a simple mass-law equation for the combination of manganese with the enzyme protein. It is of interest that, at 40° C. and pH 8, the unactivated protein was found to be more stable than the active enzyme.

The fact that extracts of swine intestinal mucosa contain a great variety of peptidases has been noted previously (7). Among

these enzymes there was found a peptidase which hydrolyzed *l*-leucylglycylglycine but whose activity, in contrast to the leucine aminopeptidase mentioned above, was not increased upon the addition of manganese ions. More recent experiments (8) have demonstrated the presence of a similar enzyme in extracts of rabbit skin and lung as well as in the sera of various animals. From extracts of rabbit skin, enzyme preparations were obtained which hydrolyzed *l*-leucylglycylglycine rapidly, and which did not hydrolyze *l*-leucinamide even in the presence of added manganese. The enzyme responsible for the hydrolysis of the tripeptide, and tentatively named "dermopeptidase," was not activated by manganese; however, the fact that it is extremely sensitive to slight acidity (pH values less than 5) leaves open the possibility that it may be a metallo-protein. The activity toward *l*-leucylglycylglycine is accompanied by activity toward other tripeptides, such as glycylglycylglycine; it has not been established, however, whether the same enzyme is involved in the hydrolysis of the two tripeptides. The results summarized above make it doubtful whether data on "aminopeptidase" activity in tissue extracts obtained with *l*-leucylglycylglycine as the substrate reflect the activity of a single enzyme. Rather, it must be concluded that tissue extracts may contain at least two different peptidases acting on this tripeptide, a manganese-activatable leucine aminopeptidase and another enzyme, of different specificity, which does not require the addition of manganese ions.

One of the peptidases present in extracts of swine intestinal mucosa, that which hydrolyzes alanylglycylglycine, has been purified extensively (9). The procedure involves precipitation with ammonium sulfate, treatment with lead acetate, reprecipitation with ammonium sulfate, and electrophoresis. The preparation thus obtained was electrophoretically homogeneous, and was essentially free of activity toward dipeptides such as alanylglycine, glycylalanine, leucylglycine, etc. Activity toward tripeptides such as glycylglycylglycine, alanylglycylalanine, or glycylleucylglycine was retained, however. It is of interest that this enzyme was readily inactivated at pH 4 and also was not activated upon addition of manganese ions. In several respects, therefore, the purified enzyme from intestinal mucosa resembles the dermopeptidase, discussed earlier.

A procedure similar to that used by Agren in the work de-

scribed above (9) was applied by him to the purification of an enzyme present in cattle muscle and which also hydrolyzes alanylglycylglycine (10). Other investigators (11) report that leucylglycylglycine is rapidly hydrolyzed by extracts of dog muscle.

The widespread distribution, in mammalian tissues, of peptidases with similar properties raises the possibility that they may have a common origin, and that they are obtained on extraction of a particular tissue such as skin or lung, not from the characteristic cells of that tissue, but from invasive cells such as leucocytes or lymphocytes, which are also known to contain active peptidases.

A careful histochemical study of the distribution, in swine intestinal mucosa, of peptidase activity toward alanylglycylglycine and alanylglycine has been performed (12). The activity for both substrates was found to increase on passing through the layers of the mucosa, the highest values being noted in the layer containing chief cells and interstitial tissue.

The suggestion has been made (13) that the intestinal peptidase acting on alanylglycylglycine is identical with the "intrinsic factor" of Castle since on one-hundred fold purification of the enzyme, the "intrinsic factor" activity also increased to a similar extent.

An ingenious method for the demonstration of peptidase activity has been described (14), in which advantage is taken of the presence of potent *l*-amino acid oxidases in the venom of snakes such as *Vipera aspis*. The "ophio-oxidase" does not act on peptides, but if a peptidase is present which releases *l*-amino acids, these are oxidatively deaminated by the venom oxidase as fast as they are set free by the peptidase. The extent of oxygen uptake (determined manometrically) thus provides a measure of the peptidase action, which is markedly accelerated by the rapid oxidative removal of the liberated amino acids. This method cannot be used if glycine is the sole amino acid liberated since the ophio-oxidase does not deaminate this amino acid. By means of this procedure it has been demonstrated that mammalian nerve tissue has appreciable enzymatic activity toward peptides such as glycyl-*l*-leucine, glycyl-*l*-tyrosine, *l*-leucylglycine, and *l*-leucylglycylglycine. In this manner, it has been shown also that tubercle bacilli contain *l*-peptidases capable of hydrolyzing the above peptides (15). Activation by manganese ions was noted, especially in the case of the tripeptide.

It may well be expected that variations of this method will be developed in which some of the specific bacterial amino acid decarboxylases are used for following the enzymatic action of exopeptidases which liberate *l*-amino acids. It will be recalled that a number of years ago, a method was devised (16) in which *d*-amino acid oxidase is used to follow the enzymatic hydrolysis of *d*-peptides.

A study of the peptidases present in glycerol extracts of rabbit bone has shown the presence in such extracts of enzymes which hydrolyze dipeptides such as glycylglycine and leucylglycine as well as tripeptides such as leucylglycylglycine (17).

*Carboxypeptidases.*—Further study of the specificity of crystalline pancreatic carboxypeptidase has shown (18) that several carbobenzoxyglycylamino acids differ greatly in their sensitivity to enzymatic action, depending on the nature of the terminal amino acid present in the substrate. Thus, to achieve comparable rates of hydrolysis of carbobenzoxyglycyl-*l*-alanine and carbobenzoxyglycyl-*l*-phenylalanine, about 340 times as much enzyme was required for the alanine-containing substrate as was necessary for the phenylalanine-containing substrate. This variation in sensitivity cannot be attributed to the presence, in the enzyme preparation, of several peptidases, since partial inactivation of carboxypeptidase results in a parallel decrease in enzymatic activity toward all substrates studied.

In the course of the work reported by Stahmann *et al.* (18) it was found also that the addition of carbobenzoxyglycylamino acids which were resistant to carboxypeptidase action (e.g., carbobenzoxyglycyl-*d*-phenylalanine, carbobenzoxyglycylglycine) would inhibit strongly the enzymatic action on sensitive substrates such as carbobenzoxyglycyl-*l*-phenylalanine. The inhibition of the hydrolysis of the *l*-substrate by the corresponding *d*-antipode is yet another example which points to the dangers inherent in quantitative enzyme studies in which racemic compounds are used as substrates.

It has been found (19) that phenylpyruvyl derivatives of amino acids (e.g., phenylpyruvyl-*l*-phenylalanine) are hydrolyzed by crystalline pancreatic carboxypeptidase. This confirms the conclusion reached in earlier studies (20) that the hydrolysis of keto acyl amino acids such as pyruvyl-*l*-phenylalanine is due to carboxypeptidase action.

Crystalline carboxypeptidase has been reported (21) to inactivate pepsitensin and hypertensin (angiotonin) readily, but no inactivation of vasopressin or oxytocin was noted even with 5000 times as much enzyme. In this connection, the study of the action of crystalline carboxypeptidase and of other proteolytic enzymes on the tetrapeptide tyrosyllysylglutamyltyrosine (22) is worthy of note. This peptide was synthesized in the hope of obtaining a material of known structure which would exhibit the pressor effect of angiotonin. Although the synthetic peptide did not show such an effect, the rate of its hydrolysis by carboxypeptidase and by pepsin was similar to the rate of inactivation of angiotonin by these enzymes. Chymotrypsin and trypsin, however, hydrolyzed the tetrapeptide much more slowly than they inactivated angiotonin. In general, the behavior of the peptide to the action of these four proteolytic enzymes agreed with current views concerning their specificity of action.

The possibility has been raised (23) that the group of enzymes named "conjugases," which split pteroyl heptaglutamate (vitamin $B_c$ conjugate) to yield pteroyl glutamic acid, are carboxypeptidases. This conclusion is based on the observation that the conjugases do not liberate pteroyl glutamic acid from the methyl ester of the conjugate (24). Conjugase activity has been obtained from a variety of tissues, including swine kidney (25) and rat liver (26). These tissues had previously been found to contain enzymes which are homospecific with pancreatic carboxypeptidase (27).

*Prolidase.*—It has been shown (1, 7) that extracts of swine intestinal mucosa contain an enzyme (prolidase) specifically adapted to the hydrolysis of peptide linkages involving the imino nitrogen of proline and hydroxyproline. Attention has been called to the presence of prolidase activity in extracts of rabbit skin and lung (8). As in the case of the enzyme from intestinal mucosa, the prolidase activity of extracts of these tissues is markedly enhanced upon the addition of manganese ions.

*Dehydropeptidases.*—It had been found in 1932 that peptide linkages involving the amino group of aminoacrylic acid (dehydroalanine) or of aminocinnamic acid (dehydrophenylalanine) were hydrolyzed by extracts of several animals tissues (28). More recently it has been shown (29) that while chloroacetyl dehydroalanine is split by extracts of liver, kidney, and pancreas of rats, mice, rabbits, and guinea pigs, this substrate is not split appreci-

ably by the sera of these animals or by extracts of spleen, brain, muscle or tumors from these species. On the other hand, glycyl-dehydroalanine was found to be hydrolyzed by extracts of all the above tissues (30). For this reason it has been suggested that there are at least two dehydropeptidases, the one acting on glycyl-dehydroalanine being designated dehydropeptidase I and the one acting on chloroacetyl dehydroalanine being designed dehydropeptidase II. Attention has been called (31) to the finding that the distribution of the enzyme responsible for the initial desulfuration of diglycylcystine and dichloroacetyl cystine is similar to that of dehydropeptidase II.

## Endopeptidases

*Pancreatic trypsin.*—It had been shown in 1941 (32) that, in the autocatalytic transformation of trypsinogen to trypsin, the formation of "inert" protein could be prevented completely by allowing the activation to proceed in the presence of calcium ions. A recent report (33) employs this finding for the improvement of the yield and quality of the crystalline trypsin prepared from trypsinogen.

The effect of crystalline trypsin in the conversion of phosphorylase *a* to phosphorylase *b* (34) has been further studied (35). The rate of conversion at pH 6 to 6.5 does not accord with first order kinetics and is not proportional to the trypsin concentration. The addition of manganese ions is without effect. This behavior is different from that observed for the PR ("prosthetic group removing") enzyme present in muscle extracts and which also converts phosphorylase *a* to phosphorylase *b*. The kinetics of the conversion by the PR enzyme follow the equation for a first order reaction over a considerable range of phosphorylase concentration. The addition of manganese ions markedly accelerates the action of the PR enzyme. The nature of the prosthetic group split off from phosphorylase *a* by trypsin or the PR enzyme has not been established as yet.

A study of the effect of preparations of mushroom tyrosinase on trypsin, as well as other protein-splitting enzymes (pepsin and chymotrypsin), indicates that the oxidases attack these proteolytic enzymes at the tyrosyl residues (36). In contrast to the action of most chemical reagents which attack the tyrosyl residues of proteins, however, the tyrosinases do not decrease the proteolytic

activity of trypsin, pepsin, or chymotrypsin. It has been suggested that the alteration undergone by the proteinases in the presence of tyrosinases is less extensive than in experiments with chemical agents.

*Trypsin inhibitors.*—A detailed description has been given of the isolation from soybean meal of a crystalline globulin which strongly inhibits the proteolytic action of trypsin (37). The inhibitor is extracted from defatted soybean meal with 0.25 N sulfuric acid and the adsorbed on bentonite. After elution with pyridine, followed by dialysis, the protein is precipitated at pH 4.65 at 5 to 10°. Crystallization is effected at pH 5.1 and 36°.

Both crude and crystalline preparations of the soybean inhibitor of pancreatic trypsin retard the coagulation of blood and also inhibit the proteinase of plasma (38).

The crystalline inhibitor obtained from pancreas has been found (39) to exhibit, in moderate concentration, antibacterial activity toward hemolytic streptococci, *S. aureus*, and *E. coli.* This effect has been interpreted as due to the inhibition of proteolytic enzymes concerned with bacterial growth.

The presence of the proteolytic inhibitor in soybean has been related to the retarding effect of unheated soybean meal upon the growth of chicks (40). Evidence has been presented for the view that the inhibition of intestinal proteolytic activity is responsible for an inadequate supply of nutritionally essentially amino acids, especially methionine. It has been reported that the autoclaving of soybean meal proteins at about 100° increases the rate of the hydrolysis of these proteins by pepsin and other crude preparations of proteolytic enzymes (41). Attention has been drawn (42) to the finding that methionine is liberated at a slower rate than are lysine and leucine during the pancreatic digestion of soybean protein, and that heat processing of the soybean meal increases the rate of enzymatic liberation of methionine to a relatively greater extent.

*Pepsin.*—Calorimetric studies have been performed on the rate of the heat absorption which accompanies the inactivation of crystalline swine pepsin near pH 6 (43). It was found that the kinetics of the heat effect are the same as for the loss of peptic activity on hemoglobin, and it has been inferred, therefore, that the rate-controlling reaction is the same for both phenomena. At 25° and pH 6.36, the heat absorption amounted to 33,500

calories per mole of pepsin. Over a narrow pH range, the heat absorption was found to vary linearly with pH, with a positive slope of 49,000 calories per mole per pH unit at 15° and 88,000 calories at 25°. Although exact interpretation of these data is difficult, it has been suggested that these large slopes cannot be explained by a change in the number of ionization constants of the ionizable groups in the pepsin molecule. In this connection, reference should be made to the older data (44) on the kinetics of pepsin denaturation, discussed in a previous volume of this series (45).

No significant effect upon the proteolytic activity of crystalline swine pepsin was noted (46) following exposure of the enzyme to seven atmospheres of oxygen for two hours at 38°.

Experiments have been reported (47) on the effect of the addition of amino acids, hydrogen cyanide, or pyrophosphate on the proteolytic activity of partially purified (non-crystalline) preparations of pepsin. The addition of M/60 alanine to enzyme solutions of 0.25 to 1 per cent caused a slight inhibition, while with 0.015 per cent pepsin, M/60 alanine markedly increased the rate of proteolysis. Similar activation was noted upon the addition of glutamic acid or histidine and analogous effects were also observed upon addition of hydrogen cyanide or pyrophosphate to pepsin. Here again, for low concentrations an activation was noted, while the reverse was true for more concentrated enzyme solutions. In view of the undefined nature of the enzyme material employed in these studies, an interpretation of the data seems hazardous.

It has been known that bovine serum globulins may be split by pepsin at pH 4.0 into particles one-half the size of the original proteins (48). A more recent study (49) has provided similar results with human gamma-globulin. The largest yield of half-molecules (sedimentation constant $s_{20} = 5.8$ S) was obtained when the digestion was performed at pH 3.5 for three days at 1 to 4°. The globulin preparations thus produced retained the diphtheria and streptococcus antitoxins, and anti-influenza A activity. The typhoid "O" agglutinin was lost in the course of pepsin treatment but the concentration of the "H" agglutinin remained unchanged.

The purification and crystallization of rennin (50) has settled the controversy as to the possible identity of this enzyme with pepsin. Additional evidence for the difference between these two

enzymes has been provided by experiments in which they were adsorbed on foam (51). Fractionation by this method yielded preparations of pepsin which were seven to eight times more active than the original commercial pepsin, while the rennin activity was reduced from 60 to 68 per cent. A study has been made of the phenomenon of rennet hysteresis, in which the time of coagulation of heated milk is progressively greater with increase in the time interval between heating and the addition of rennet (52). This result is apparently not due to an effect of the enzyme but rather to the adsorption of calcium phosphate by the calcium caseinate-calcium phosphate complex.

*Intracellular plant proteinases.*—In a recent note (4), it has been suggested that the proteinases of the higher plants be separated into two classes, depending on their activation behavior. The members of one group, which includes papain, ficin, and bromelin, which may be inactivated by oxidizing agents and, under certain conditions, reactivated by reducing agents, have been termed "anastrophic" proteinases. A second group, including solanain, hurain, and arachain, have been termed "stasidynic" proteinases because they are not affected by certain mild oxidizing or reducing agents. It may be questioned whether this nomenclature will gain general adherence in view of the conflicting experimental data on the activation properties of the plant proteinases. In large part, the current state of uncertainty is due to the fact that crude enzyme preparations have been used and the effect observed on addition of oxidizing or reducing substances may well have been the result of the action of these agents on natural activators or inhibitors present in the enzyme preparations (53).

It has been reported (54) that papain and related plant proteinases (chymopapain, ficin, bromelin, etc.) may be activated by thiosulfate; in these experiments, however, crude enzyme materials were employed and the possible intervention of natural activators has not been excluded.

It has been reported (55) that crude papain is inhibited by certain war gases and vesicants, such as chloropicrin, chloroacetophenone, chloroacetone, and bis (chloroethyl)sulfone. This effect has been attributed to a combination of these agents with sulfhydryl groups which are present either in the accompanying natural activators or in the enzyme itself.

Advantage has been taken of the chemical specificity of papain in developing a new synthesis of *l*-glutamine (56). In this method, carbobenzoxy-*l*-glutamic acid diamide is treated with cysteine-activated papain to yield carbobenzoxy-*l*-glutamine, which, upon catalytic hydrogenation, is converted to the amino acid.

A study of the action of crude papain or bromelin on human gamma-globulin (57) has shown that, at pH 5, the activated enzymes split the protein into particles one-fourth the size of the original globulin particle. In the ultracentrifuge, a single boundary corresponding to a sedimentation constant $s_{20}$ of 4.1 S was found for the split products. Electrophoretic analysis of the digests indicated the presence of a number of separate components which also were found to differ in their solubility in ammonium sulfate and ethyl alcohol.

The properties of a proteinase present in the latex of *Euphorbia lathyris* have been described recently (58). The enzyme ("euphorbain") exhibits maximum reduction of gelatin viscosity at pH 8 and, like solanain, is not activated upon addition of sulfhydryl compounds. The addition of iodoacetic acid or various metallic ions, however, causes inhibition of enzymatic activity.

The effect of heat on the proteinase activity of native flour has been examined (59) and it was found that, in dilute acetic acid, the temperature of inactivation was above 70°, while optimal enzymatic action was noted at about 40°. The proteinase in aqueous extracts of wheat and its milled fractions has a pH optimum (60) of about 5. It has been noted that *n*-octyl alcohol, sodium fluoride and ultraviolet radiation inhibit the proteolytic activity. However, the proteolytic activity of a straight flour on casein is not inhibited by 0.001 to 0.01 per cent potassium bromate (61). This result has been interpreted as additional evidence against the view that the action of bromate in dough involves the proteolytic enzyme of flour.

*Intracellular animal proteinases.*—Despite the demonstration several years ago (62) that extracts of animal tissues contain a number of peptide-splitting enzymes which may be classified as endopeptidases, little progress has been reported thus far in the separation and purification of these enzymes. The possible role of the intracellular proteinases (frequently termed "cathepsin") in the metabolic synthesis of peptide bonds has been discussed in recent articles (63, 64) and emphasis has been placed on the need

for the study of coupled reactions in which energy is provided for the reversal of the hydrolytic action of these enzymes. It has been stated by some authors [cf. (65)] that the biosynthesis of a peptide bond cannot involve the reversal of its hydrolysis, on the grounds that energy is required for the synthetic process; it is frequently overlooked, however, that there are numerous cases in which living systems may effectively counteract the thermodynamic tendency of chemical compounds to decompose by coupling the reaction to another energy-yielding reaction. An excellent example is provided by the recent work on the enzyme-catalyzed fixation of carbon dioxide by alpha-ketoglutaric acid (66). It must be reiterated also that, in the case of the intracellular synthesis of peptide bonds, any proposed scheme of reactions must take into account the extremely precise specificity of action required for the reproducible synthesis of the complex protein molecule. Thus far, the only tissue enzymes which are known to exhibit the requisite structural specificity are the intracellular proteolytic enzymes.

Pending efforts to clarify the metabolic role of the intracellular proteinases, studies have been reported recently on the proteolytic activity of these enzymes in extracts of a variety of normal and neoplastic tissues. In one of these studies (67), the catheptic activity of extracts of spontaneous and induced mouse hepatomas was measured using hemoglobin as the substrate, and the results indicated a higher activity in extracts of the neoplastic tissues than in extracts of normal mouse liver. In another study (68), the protein substate was replaced by benzoyl-*l*-argininamide, previously shown to be hydrolyzed by one of the intracellular proteinases (62), and essentially similar results were obtained as reported by Maver *et al.* (67).

Determinations of the proteolytic activity of glycerol extracts of thyroid tissue, using edestin as the substrate, have shown (69) that in severe goiter, the rate of splitting per unit weight of tissue was nearly double that found for normal thyroid. It has been inferred that the intracellular proteinases split the thyroglobulin into diffusible fragments, and that the proteolytic action of these enzymes is inhibited when iodine is administered.

Glycerol extracts of rabbit bone have been found to exhibit proteolytic activity toward gelatin, beef serum proteins, and denatured hemoglobin (70). The enzymatic activity is optimal at

pH 4 and is increased upon the addition of sulfhydryl compounds, cyanide, manganese, cobalt, or zinc.

It has been suggested that the proteolytic activity of malignant tissue cells, to which has been attributed the liquefaction of culture media used for tissue culture studies, is responsible in part for the infiltration of malignant cells into normal tissue. Recent experiments (71) have shown that when chicken sarcoma cells were grown on rabbit plasma, no digestion took place unless fresh chicken serum was added. If the chicken serum was previously heated to 56° for three to four hours, however, no liquefaction of the plasma clot occurred. This result is explained by assuming that inactive proenzymes in homologous sera are activated by tumor cells of the corresponding species. The activation of the proteolytic enzymes of the culture medium would thus lead to the dissolution of the plasma clot.

*Plasma proteinases.*—Recent work has provided strong evidence for the view that the well-known proteinase activity of plasma, previously attributed to an enzyme variously termed "plasma (or serum) trypsin," "plasma (or serum) tryptase," or "plasma (or serum) protease," is not due to the action of pancreatic trypsin. It is generally agreed, therefore, that the designation of this enzyme as a "trypsin" is undesirable. The term "plasmin" has been suggested (72) and appears acceptable pending efforts to define the enzymatic homogeneity and specificity of the proteinase, as well as its source in the tissues. Although plasmin and trypsin have been found to be similar with respect to several properties such as pH optimum, inhibition by various substances such as heparin (73), and clotting effect on normal and hemophilic blood (74), the difference between the enzymes is clearly established by the failure of plasmin to attack a typical substrate of trypsin, benzoyl-*l*-argininamide, and by the demonstration that trypsin is capable of hydrolyzing peptide linkages in casein which are apparently resistant to the action of plasmin (72). The nature of the peptide linkages specifically hydrolyzed by plasmin has not been established, however. It has been noted recently (8), that rabbit serum contains an endopeptidase which is able to hydrolyze benzoylglycinamide. The possible identity of this enzyme with plasmin is a matter for future investigation.

The fact that plasmin may be present in the blood in an inactive state has long been recognized. It has been shown recently,

however, that the activation of the proteinase may be accomplished by a proteolytic enzyme present in hemolytic streptococci and termed streptokinase (72, 75). The term "fibrinolysin" still used to describe this bacterial enzyme should be abandoned, since the lysis of fibrin clots effected by the addition of streptokinase is apparently due to the action of activated plasmin. Recently, it has been shown (76) that streptokinase does not activate trypsinogen, and that enterokinase, which does activate trypsinogen, does not activate the plasmin precursor "plasminogen." Additional evidence is thus provided for the dissimilarity between plasmin and trypsin.

In a recent report (77), the phenomenon of shock associated with the injection of peptone into dogs has been attributed to the activation of plasma proteinase by substances released during the disintegration of platelets.

A comparative study of the hypertensinase activity and proteinase activity of plasma has been reported (78). It was noted that the hypertensinase activity bore a close relation to the action on *l*-leucylglycine. On heating the plasma to 58° and acidifying to pH 3.2, there resulted a parallel decrease in the hypertensinase and aminopeptidase activity. The authors reiterate the view that hypertensinase is related to an aminopeptidase of plasma.

The study of the plasma proteinases is made difficult by the fact that they are strongly inhibited by a variety of substances present in blood. It has been known that this inhibition may be released to a considerable degree by treatment with chloroform. In this manner, the proteinase activity of fractions of human plasma has recently been determined (79).

The fact that plasma contains substances which will inhibit the proteolytic action of enzymes such as trypsin has long been recognized. A recent study has shown that the plasma of humans and rats is strongly inhibitory to a skin proteinase (80). Fractionation of plasma with ammonium sulfate indicates that the inhibitor is associated with the albumin fraction. In another investigation (81), it has been suggested that the antiproteolytic activity of serum toward the proteolytic enzymes of leucocytes may be due to the presence of reducing substances, and especially cysteine-containing polypeptides.

The question has been raised (82) as to whether the gradual

disappearance of skin proteinase in burned skin, noted in earlier studies (83), is due to the entrance of an inhibitor into the tissue or to a loss of enzyme from the skin. Experiments designed to elucidate this matter have shown that extracts of burned skin exhibit no appreciable inhibitory action on the proteinase activity of extracts of normal skin. It has been suggested, therefore, that the decrease in skin proteinase after burning is due mainly to the escape of the enzyme into the circulation.

It has been reported (84) that, in the course of pregnancy, the "fibrinolytic" activity of blood serum may be markedly increased when complications arise.

*Bacterial proteinases.*—Since the discovery, in the serum of animals treated with hemolytic streptococci, of a factor which counteracts the dissolution of blood clots observed on addition of streptococcal filtrates (85), much attention has been given to the enzymatic factors involved in this phenomenon. As noted in an earlier section of this review, it is now considered that the material in the bacterial extracts, named "fibrinolysin" or preferably "streptokinase," activates an inactive precursor of a plasma proteinase which, in turn, acts on the fibrin of the clot. Appreciable concentration of streptokinase has been effected (86) by a series of operations involving fractional precipitation of the enzyme from culture filtrates by means of ammonium sulfate and alcohol, followed by adsorption on alumina. A recent study (87) of the antibody elicited following infection with hemolytic streptococci has shown that it readily inhibits the action of streptokinase in initiating the dissolution of fibrin clots. The "antifibrinolysin" or "antistreptokinase" appears to be quite specific and does not affect the activity of the serum proteinase. A method has been devised for the measurement of the antistreptokinase level in sera (88).

Examination of the proteolytic activity of thermophilic bacteria has shown that cell-free filtrates of *B. thermophilus*, *B. aerothermophilus*, and *B. thermoacidurans* split gelatin optimally at pH values near 8 and casein at pH 7.5 (89). When these organisms were grown in the presence of carbohydrate, the proteolytic activity of the culture filtrates was decreased but the formation of intracellular proteinases was not inhibited (90).

Filtrates from old cultures of *Clostridium tetani* have been

found (91) to possess proteolytic activity toward gelatin at pH 7. No correlation was noted between the proteinase activity and the toxin content of culture filtrates.

The production of proteinase activity by *Bacillus subtilis* is favored by the presence of manganese ions during growth (92). It would appear that the manganese functions during the elaboration of the enzyme rather than merely serving as an activator of the proteinase system.

## Proteolytic Enzymes and Protein Structure

The value of the protein-splitting enzymes as tools for the study of protein structure has received continued interest. Unfortunately, much of the experimental effort in this direction has involved the use of proteinase preparations of poorly defined enzymatic homogeneity. There can be no doubt, however, that the continued exploration of the relationship between the specificity of proteinases and the nature of the peptide bonds which they split in proteins will yield important data on protein structure.

In a study of the mode of action of papain and pepsin on various proteins (93), use has been made of the observation that peptides of leucine and valine, whose free amino group is supplied by either of these amino acids, yield appreciable quantities of acetone on oxidation with chromic acid. The acetone obtained was, however, less than the amount formed upon oxidation of the corresponding free amino acids. On the other hand, when leucine or valine was at the carboxyl end of the peptide, the amount of acetone which was formed was very small. From data secured following the action of cyanide-activated papain on casein, it has been concluded that there are liberated, in the course of enzyme action, appreciable quantities of free leucine and valine and, in addition, peptides containing these amino acids. With casein as substrate, pepsin has been found to liberate peptides of leucine and valine. It has been inferred that much of the leucine in casein is situated at terminal positions in peptide chains while this is not thought to be true for valine. This approach has been extended to the study of the action of papain, pepsin, and pancreatin on edestin and several globins (94).

A study of the enzymatic hydrolysis of zein (95) has led to the conclusion that there occurs first a splitting of linkages, possibly

the disulfide bonds of cystine, which leads to the appearance of sulfhydryl groups. With pepsin, the subsequent hydrolysis resulted in the formation of products which were, on the average, tetrapeptides. In these peptides, the most frequent terminal amino acid was reported to be glutamine.

Much work has been done in previous years on the phosphopeptone obtained upon treatment of casein with pancreatin. The introduction of the periodate method for the estimation of hydroxyamino acids (96), has permitted a closer investigation of the chemical nature of this caseopeptone (97). The material appears to be an octapeptide which contains one molecule of serine, two of phosphoserine, two of isoleucine, two of glutamic acid, and one of an unidentified component. Additional proof has been provided for the structure of the dipeptide obtained on partial hydrolysis of the peptone and strong support has been given for its formulation as phosphoserylglutamic acid.

A study of the combined action of crystalline pepsin, trypsin and chymotrypsin on raw and heated casein has shown (98) that the rate of digestion did not differ greatly for the two protein preparations. However, the amount of available lysine liberated, as determined with bacterial lysine decarboxylase, was much less in the case of the casein which had been heated to 150°.

It is of interest that ferritin, the iron-protein obtained from several mammalian tissues, is not attacked by pancreatin or papain and that the iron remains firmly attached following enzymatic treatment (99).

Experiments of fundamental significance for the study of protein structure and the mechanism of enzymatic activity have been performed (100) in which crystalline trypsin and pepsin were shown to act on a protein film through a screen of inert material such as a formaldehyde-polyvinyl polymer (Formvar). It would appear from these results that proteolytic enzymes may act on their substrates at distances greater than 100 Å.

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# THE CHEMISTRY OF THE CARBOHYDRATES

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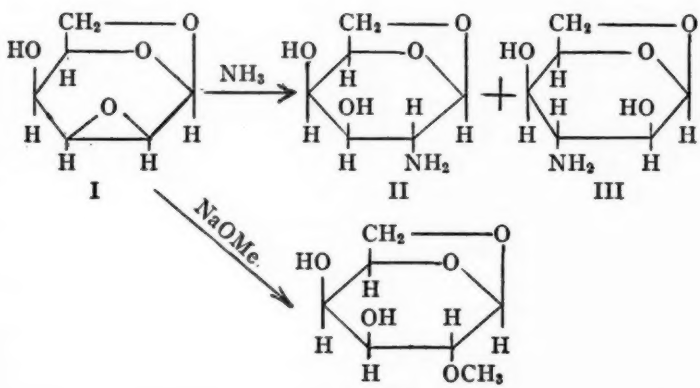
The output of publications on carbohydrates has been so great that the reviewer has been obliged to omit many topics of interest such as the studies of the hexitols and their derivatives, the osazones and the synthesis of nucleosides, and certain important desoxy sugars, to mention but a few examples.

## Amino Sugars

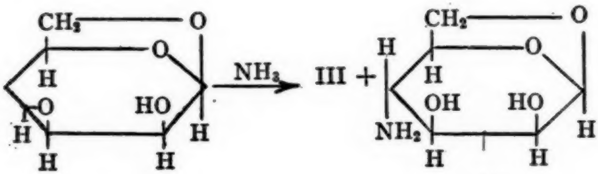
The outstanding advance in this field has been the establishment of the constitution of chondrosamine as 2-amino *d*-galactose by James, Smith, Stacey & Wiggins (1). Starting with 2,3-1,6-dianhydro $\beta$-talose (I), treatment with ammonia gave 2-amino 1,6-anhydro $\beta$-galactose (II), (isolated as the hydrochloride, $[\alpha]_D^{22°}-15.5°$ in 56 per cent yield) and a small yield of 3-amino 1,6-anhydro $\beta$-idose (III) (as the acetamido diacetate, m.p. 245–6°). Treatment of I with sodium methoxide gave 2-methyl 1,6-anhydro $\beta$-galactose (IV) (isolated as its monoacetone derivative in 60 per cent yield) and no idose derivatives could be isolated.



I II III

IV

The constitutions of II and III were proved by examining the action of ammonia on 3,4-1,6-dianhydro $\beta$-talose (V), which gave two products, a crystalline hydrochloride, $[\alpha]_D^{18°}-100°$ in 80 per cent yield, and the same acetamido anhydrohexose, m.p. 245–6° which had been obtained from I. The products to be expected from V are clearly 3-amino 1,6-anhydro $\beta$-idose (III) and 4-amino 1,6-anhydro $\beta$-mannose (VI), and from I, 2-amino 1,6-anhydro $\beta$-galactose (II),



V VI

and 3-amino 1,6-anhydro $\beta$-idose (III). This latter substance is common to both sets of reactions and is clearly the substance isolated in small yield as the crystalline acetamido diacetate, m.p. 245–6°, so that the crystalline hydrochloride $[\alpha]_D^{22°}-15.5°$, isolated in the first experiment must be II.

Fission of the 1,6-anhydro ring in II with concentrated hydrochloric acid, a process which cannot effect a configurational change except on $C_1$, gave 2-amino galactose hydrochloride which was identical in physical properties, including the x-ray powder photograph, with a specimen of chondrosamine hydrochloride prepared previously from bovine tracheal cartilage (2). Furthermore the product obtained on acetylation (m.p. 238°) was identical with $\beta$-pentaacetyl chondrosamine.

The amide of 2,3,4-trimethyl $\alpha$-methylglucuronoside and 3,4,6-trimethyl N-acetyl $\alpha$-methylchondrosaminide were isolated from the products of hydrolysis of sulphate-free methylated degraded chondroitin sulphate (3).

An independent confirmation that chitosamine (glucosamine) is pyranose in form and is 2-amino *d*-glucose has been secured by the x-ray studies of Cox (4). By the methylation and subsequent hydrolysis of N-acetyl $\alpha$-methylglucosaminide Neuberger (5) has prepared N-acetyl 3,4,6-trimethyl $\alpha$-methylglucosaminide thus proving the pyranoside structures of both the $\alpha$- and $\beta$-methyl-

glucosaminides and of N-acetyl glucosamine, since the product obtained was identical with the compound previously prepared by Cutler, Haworth & Peat (6) from β-methylglucosaminide and as synthesised from 4,6-dimethyl 2,3-anhydro-β-methylmannoside by Haworth, Lake & Peat (7) in their proof that chitosamine was 2-amino *d*-glucose. The oxidation of 3,4,6-trimethyl glucosamine hydrochloride with two equivalents of naphthalene *i*-sulphonchloramide gave 2,3,5-trimethyl *d*-arabofuranose, which was characterised as the crystalline lactone and amide (5).

3-Methyl glucosamine and 3-methyl glucosamic acid have been prepared from N-acetyl 4,6-benzylidene α-methylglucosaminide (8), and the assigned constitution confirmed by periodate oxidation. N-acetyl 3-methyl α-methylglucosaminide is not attacked by periodic acid indicating the absence of adjacent hydroxyl groups, so that the substituent must be at $C_3$ or $C_4$. 3-Methyl glucosamic acid, however, takes up two atoms of oxygen with the formation of one molecule each of formaldehyde and formic acid, no ammonia being liberated. It follows that there is no free hydroxyl residue adjacent to the amino group and that three free hydroxyl groups are contiguous.

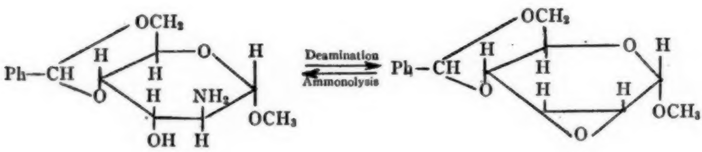
Neuberger & Pitt Rivers (9) have hydrolysed N-acyl β-methyl-glucosaminides by an enzyme extract from *Helix pomatia* which is specific for the N-formyl and -acetyl derivatives of these β-glycosides, but is unable to effect the hydrolysis of acyl derivatives of higher fatty acids or nonacetylated or completely O-substituted glucosaminides. Calculations from molecular rotations lend support to the view that in chitobiose, chitotriose, and chitin the 2-amino glucose residues are united by β-linkages.

From their experiments with barium acid heparinate Wolfrom *et al.* (10) consider that the nitrogen atoms of the glucosamine component of the substance are neither acetylated as in mucoitin sulphate and the ovomucoid polysaccharide (11), nor free, and are engaged in polymeric linkages of an unusual type.

Streptomycin includes *l*-glucosamine in its building stones since N-methyl *l*-glucosamine pentaacetate has been isolated from streptobiosaminide dimethyl acetal by hydrolysis followed by acetylation (12). The constitution was proved by the isolation of *l*-glucosazone from the free base and its conversion into the corresponding *l*-glucosephenylosotriazole. The free hexosamine on oxidation gave the enantiomorph of N-methyl *d*-glucosamic acid;

the aminohexose concerned was synthesised from *l*-arabinose, methylamine, and hydrogen cyanide; and the enantiomorph of the N-methyl hexosamine pentaacetate was prepared from *d*-glucosamine.

Wiggins has made the important observation (13) that the methylglycosides of amino sugars can be deaminated by nitrous acid to give anhydrides; thus 2-amino 4,6-benzylidene α-methyl-altroside (VII) gives 2,3-anhydro 4,6-benzylidene α-methylal-loside (VIII) which is reversibly converted into VII by treatment with ammonia. Deamination therefore is accompanied by Walden inversion on the carbon atom originally carrying the amino group.



VII VIII

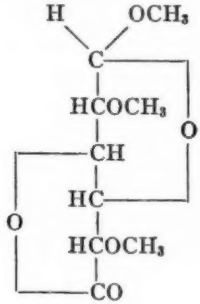
N-acetyl *d*-glucofuranosylamine has been obtained by the action of ethanolic ammonia on β-*d*-glucose pentaacetate (14). This is somewhat disturbing in view of the frequent use of alcoholic ammonia for deacetylation in the sugar series.

## Uronic Acids

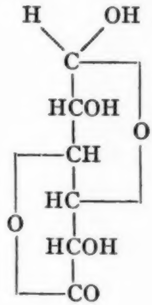
*d*-Glucuronic acid would be expected to exist mainly in the pyranose form and lactonisation to take place by the elimination of water between the hydroxyl groups on $C_6$ and $C_3$, since the formation of a δ-lactone is stereochemically impossible in this case. The work of Smith (15) shows, however, that trimethyl glucurone is 2,5-dimethyl α-methylglucofururonoside (IX) and confirms the earlier suggestion of Reeves (16). This is perhaps hardly surprising in view of the fact that 3,6-anhydro methylglucopyranosides are readily converted into the more stable furanosides, showing that in the case of glucose two five-atom rings are more stable than one five- and one six-atom ring (17). The proof in the present case depends on the fact that trimethyl glucurone on methylation, oxidation and esterification gave 2,3,5-trimethyl

glucosaccharo-1,4-lactone 6-methyl ester previously synthesised from glucose via 6-trityl methylglucofuranoside and 2,3,5-trimethyl glucose (18). To *d*-glucurone, therefore, the formula X is ascribed.

Wolfrom & Rice (19) have established the uronic acid com-



IX



X

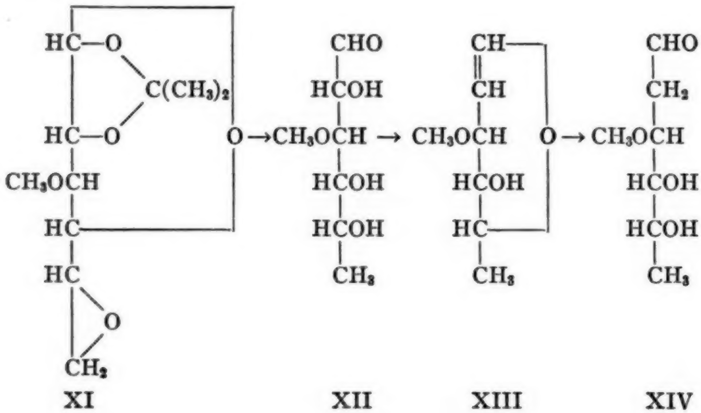
ponent of heparin as *d*-glucuronic acid since hydrolysis under oxidising conditions gave *d*-glucosaccharic acid.

## Sugar Anhydrides

The production of 3,6-anhydro sugars by the treatment with alkali of sugar sulphates (20) and nitrates (21) is now well established, but, until recently, no proof was available as to whether ethylene oxide rings could be produced by the hydrolysis of inorganic sugar esters. Percival & Duff (22) have now shown that barium 1,2-monoacetone 3-methyl glucofuranose-6-sulphate yields the corresponding 5,6-anhydride on treatment with sodium methoxide. The previous conclusion that 5,6-anhydrides were not produced (23), based on the failure to obtain *l*-idose derivatives from barium 1,2-monoacetone glucofuranose-6-sulphate, is now seen to be based on an unsound foundation since Reichstein *et al.* (24) have shown that 1,2-monoacetone 5,6-anhydroglucofuranose is converted into the corresponding 3,6-anhydride on treatment with alkali and not into *l*-idose derivatives, contrary to previous reports (25). It was for this reason that the experiments were repeated (22) with the hydroxyl group on $C_3$ blocked by methoxyl, which had the additional advantage of making the barium ethereal

sulphate under investigation soluble in methanol, thus making possible a smooth reaction with sodium methoxide. No anhydride formation was observed when phosphate residues were removed by alkaline hydrolysis from the barium salts of α-methylglucopyranoside-6-phosphate, methylglucofuranoside-3-phosphates and 1,2-monoacetone glucose-3- and -6- phosphate (26).

The ethylene oxide sugar anhydrides continue to be of great value for interconversion reactions. Reference has already been made to their use in elucidating the constitutions of chondrosamine and chitosamine, and Reichstein and his co-workers have employed these reactive substances in many new syntheses. Thus 1,2-monoacetone 3-methyl 5,6-anhydroglucofuranose (XI) has been converted by hydrogenation and hydrolysis into 3-methyl *d*-quinovose (XII) from which, via the corresponding glycal (XIII), 2-desoxy 3-methyl *d*-quinovose [*d*-oleandrose (XIV)] was obtained (27).



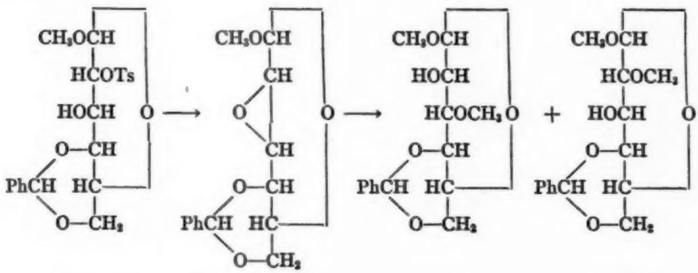
XI XII XIII XIV

*d*-Idose was obtained from *d*-galactose by the following route (28): 4,6-benzylidene α-methylgalactoside→3-tosyl 4,6-benzylidene α-methylgalactoside→2,3-anhydro 4,6-benzylidene α-methylguloside→4,6-benzylidene α-methylidoside→α-methylidoside→*d*-idose. The idose so obtained is always contaminated with *d*-idosan ⟨1,5⟩⟨1,6⟩, and the first crystalline derivative of idose to be recorded is the benzyl mercaptal (29).

The structures of the 2-tosyl 4,6-benzylidene methylga-

lactosides from which the 2,3-anhydrotalose derivatives are prepared, and of the 3-tosyl 4,6-benzylidene methylgalactosides which give the corresponding 2,3-anhydrogulose derivatives, have been unequivocally proved by conversion to 3-methyl and 2-methyl galactose respectively (30, 31).

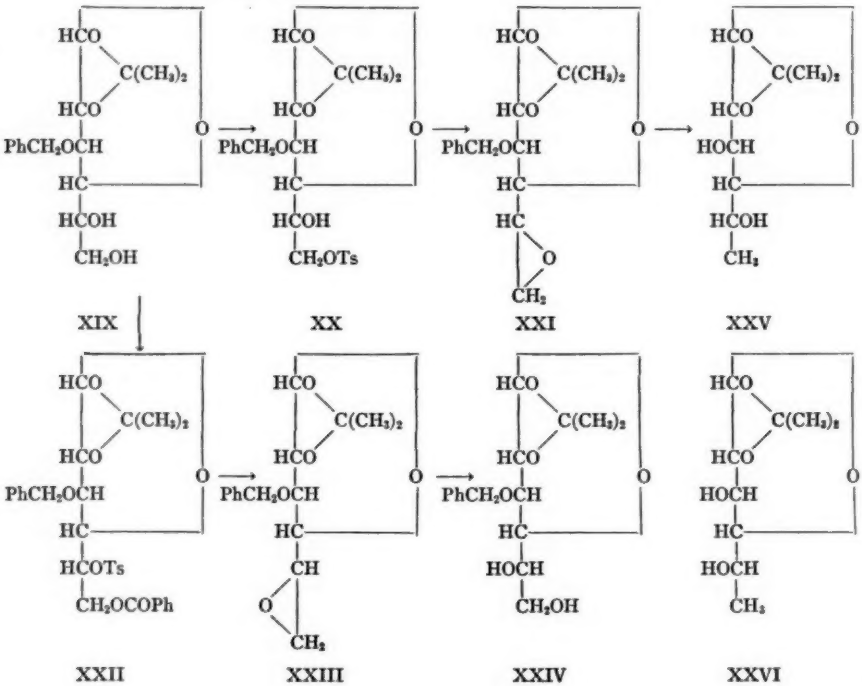
Wiggins (32) also records the conversion of galactose into *d*-idose derivatives. He converted 2-tosyl 4,6-benzylidene β-methylgalactoside (XV) into 2,3-anhydro 4,6-benzylidene β-methyltaloside (XVI) which with sodium methoxide gave 3-methyl 4,6-benzylidene β-methylidoside (XVII) and 2-methyl 4,6-benzylidene β-methylgalactoside (XVIII). This author has also recorded the very slow hydrolysis of 2-tosyl 4,6-benzylidene α-methylgalactoside with alkali, a similar though not analogous case to the behaviour of the highly resistant 3-tosyl 2,4,6-trimethyl α-methylgalactoside (33).



XV XVI XVII XVIII

Meyer & Reichstein have obtained *l*-idose from *d*-glucose as follows (34): 1,2-Monoacetone 3-benzyl glucofuranose (XIX) was converted by tosyl chloride to the 6-tosyl derivative (XX) which on treatment with alkali gave 1,2-monoacetone 3-benzyl 5,6-anhydroglucofuranose (XXI). Treatment of XIX with benzoylchloride followed by tosyl chloride gave 1,2-monoacetone 3-benzyl 5-tosyl 6-benzoyl *d*-glucofuranose (XXII) which on deacylation gave a product (XXIII) different from XXI and which is therefore designated 1,2-monoacetone 3-benzyl 5,6-anhydro *l*-idofuranose. Heating with alkali in dioxan converts XXIII into 1,2-monoacetone 3-benzyl *l*-idofuranose (XXIV), from which the benzyl group is removed by hydrogenation under pressure, and the 1,2-isopropylidene *l*-idofuranose so obtained is converted into

crude *l*-idose by dilute acid, being identified by reduction and acetylation to *l*-iditol hexacetate. In the same series of experiments pressure hydrogenation was used to convert XXI into 1,2-monoacetone *d*-quinovofuranose (1,2-monoacetone *d*-glucomethylofuranose) (XXV) and XXIII into 1,2-monoacetone *l*-idomethylofuranose (XXVI).



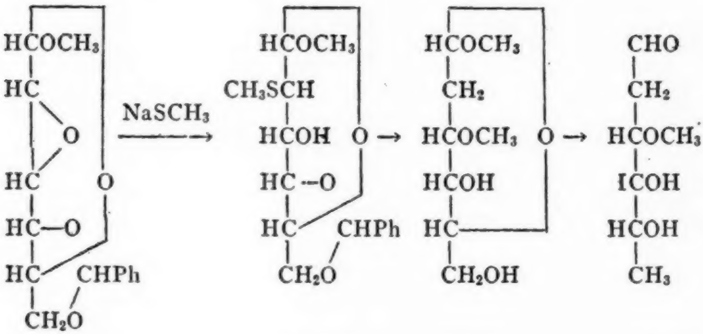
XIX XX XXI XXV

XXII XXIII XXIV XXVI

A new method for the preparation of desoxy sugars (35) depends on the conversion of ethylene oxide anhydrides into methylthiol derivatives followed by catalytic reduction. Thus 2,3-anhydro-4,6-benzylidene $\alpha$-methyl-*d*-allopyranoside (XXVII) is converted by sodium methyl mercaptide in methanol into 2-methylthiol 4,6-benzylidene $\alpha$-methyl-*d*-altropyranoside (XXVIII) which is methylated to give the corresponding 3-methyl ether. Hydrogenation gives 2-desoxy 3-methyl $\alpha$-methyl-*d*-allopyrano-

side (XXIX). XXIX has been converted into *d*-cymarose (XXX) *via* the 6-iodide in the usual way (36).

The action of alkali on phenylglycosides continues to attract attention, and McCloskey & Coleman (37) have suggested that the formation of a $\beta$-1,6-anhydride from a phenyl-$\beta$-glycoside is the result of two Walden inversions. The suggested mechanism is the elimination of the phenoxy residue with the formation with inver-

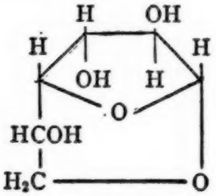


XXVII XXVIII XXIX XXX

sion of a 1,2-anhydride, which then reacts with the hydroxyl group on $C_6$ with inversion to give the $\beta$-1,6-anhydride. In support of this view it is pointed out that when 2,3-dimethyl $\beta$-phenyl-*d*-glucoside was treated with alkali no phenol was liberated and that the corresponding tetramethyl derivative was recovered unchanged. On the other hand, substitution on $C_4$ as in the phenyl-$\beta$-lactoside and -cellobioside does not inhibit 1,6-anhydride formation (38). The hypothesis requires, however, that the phenoxy group should be *trans* to the hydroxyl group on $C_2$ and it is claimed that this explains why no 1,6-anhydride is formed from the corresponding $\alpha$-phenylglucoside. The fact that $\beta$-phenyl-*d*-mannoside and $\alpha$-phenyl-*d*-galactoside on treatment with alkali yield 1,6-anhydrides, the latter albeit slowly, in both of which the hydroxyl groups on $C_2$ are *cis* to the phenoxy group makes it clear that the above mechanism is open to doubt (39).

A new glucosan (XXXI) has been isolated by the vacuum pyrolysis of starch (40). The structure 1,6-anhydro-$\beta$-*d*-glucofuranose is assigned to this substance since methylation and

hydrolysis yields 2,3,5-trimethyl *d*-glucose characterised as the crystalline phenylhydrazide of the corresponding acid, and the tritosyl derivative of XXXI is stable to sodium iodide in acetone at 100° thus excluding the presence of a primary alcohol residue. The $\beta$-configuration is assumed on steric grounds, and it is con-

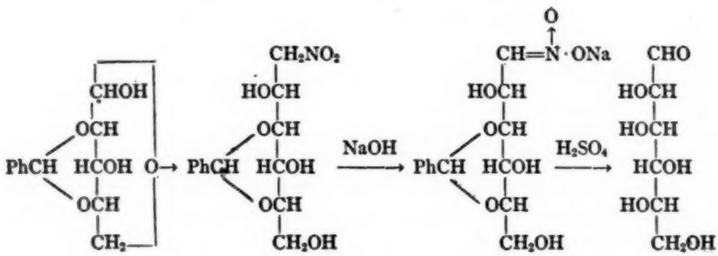


XXXI

cluded that the furanose ring is stabilised by the 1,6-ring. Rather unexpectedly, the substance does not react with periodic acid.

## Unusual Reactions

Sowden & Fischer (41) have continued their preparations of carbohydrate C-nitro alcohols and have condensed nitromethane with 2,4-benzylidene *l*-xylopyranose (XXXII) to yield 2,4-benzylidene 6-nitro 6-desoxy sorbitol (XXXIII). Treatment of the *aci*-sodium salt (XXXIV) with moderately concentrated sulphuric acid gave *l*-gulose (XXXV) (50 per cent yield). This method



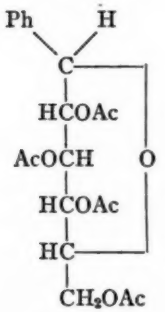
XXXII XXXIII XXXIV XXXV

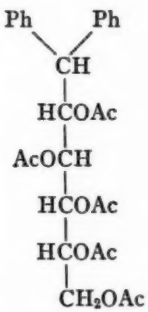
therefore provides a useful alternative to the ascent of the sugar series by the Kiliani reaction since only one isomer appears to be formed on condensation with nitromethane, no iditol derivatives being isolated along with XXXIII. A similar reaction with 4,6-

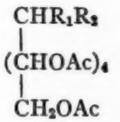
benzylidene *d*-glucose yielded 5,7-benzylidene 1-nitro 1-desoxy *d*-$\alpha$-glucoheptitol converted as above into *d*-$\alpha$-glucoheptose (42).

The diazomethane-aldonyl chloride reaction has been used by Wolfrom & Thompson (43) to prepare crystalline *l*-fructose from *l*-arabinose. It is interesting to note that this, the first crystalline specimen of *l*-fructose to be prepared, has a crystalline structure not enantiomorphous with the usual form of *d*-fructose.

By the application of the Friedel-Crafts reaction to $\alpha$-acetochloroglucose, Hurd & Bonner (44) have prepared tetraacetyl *d*-glucopyranosyl benzene (XXXVI) together with 1,1-diphenyl 1-desoxy *d*-glucitol pentaacetate (XXXVII).


XXXVI


XXXVII


XXXVIII

*p*-(Tetraacetyl *d*-glucopyranosyl) toluene and triacetyl *d*-xylopyranosyl benzene have been prepared as well as XXXVI, using the corresponding fully acetylated sugars, aluminum chloride, and the hydrocarbons (45), so that the acetochlorocompounds are not essential as starting materials, being formed *in situ*. The same workers have applied the Grignard reaction to prepare XXXVI in both $\alpha$- and $\beta$-forms, e.g., by condensing $\alpha$-acetochloroglucose with phenylmagnesium bromide followed by acetylation (46). It has also been shown that the polyacetyl glycosyl hydrocarbons condense with hydrocarbons in the presence of aluminum chloride to give the polyacetyl 1,1-diaryl 1-desoxy alditols (47) (XXXVIII).

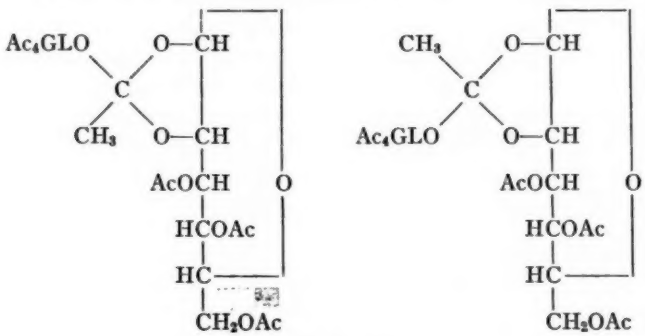
A surprising deacetylation of methyl 2,3,4-triacetyl 6-iodo 6-desoxy $\alpha$-*d*-glucopyranoside with hot aqueous-alcoholic hydrogen chloride has been recorded (48) when the acetyl residues are smoothly eliminated without affecting the glycosidic residue.

## Disaccharides

The clever constitutional synthesis of cellobiose by Haskins, Hann & Hudson (49) has been followed by a direct synthesis which is also a constitutional proof of structure by Gilbert, Smith & Stacey (50). Sodium was dissolved in molten 1,2,3,6-tetraacetyl *β-d*-glucopyranose in an atmosphere of dry nitrogen to form a 4-sodium derivative to which pure acetobromoglucose was added. After chromatographic separation, crystalline cellobiose octaacetate was isolated (10 per cent yield) and identified by comparison of its rotation, x-ray powder photograph and mixed melting point with cellobiose octaacetate prepared from cellulose. Gentiobiose octaacetate (80 per cent yield) was synthesised even more readily from the sodio 1,2,3,4-tetraacetyl *β-d*-glucopyranose.

The enzymic synthesis of sucrose has been followed by similar syntheses using glucose 1-phosphate and the enzyme of *Pseudomonas saccharophila* and *α-d*-glucopyranosido *α-l*-sorbofuranoside (51) and *α-d*-glucopyranosido *β-d*-ketoxylofuranoside (52) have been prepared.

Evans and his co-workers have made some interesting observations on the condensation of acetobromomannose with 1,2,3,4-tetraacetyl *β-d*-mannopyranose and the corresponding glucose derivative (53). Using the former compound, 6-*β-d*-mannosido-*β-d*-mannose octaacetate was obtained; but in the glucose series, in addition to the normal form of disaccharide, two crystalline disaccharide acetates to which ortho ester structures have been given (XXXIX) were isolated, two isomers being possible owing to the introduction of a fresh asymmetric centre. The presence of




XXXIX

iodine during the condensation appeared to favour the formation of the normal biose link whereas its absence favoured the formation of the ortho ester types.

In connection with this work, designed to throw light on the constitution of the *konjac* mannan, octaacetyl 6-*d*-mannosido β-*d*-glucose was converted into the crystalline acetobromo-derivative which was then condensed with the 1,2,3,4-β-tetraacetates of *d*-glucose and *d*-mannose. In this way the following crystalline trisaccharide hendecaacetates were obtained: (*a*) 12-β-*d*-mannosido-β-gentiobiose, and (*b*) 12-β-*d*-mannosido-β-*epi*-gentiobiose; the latter showed resemblances to, but could not with certainty be identified with, the trisaccharide isolated from *konjac* mannan (54).

## POLYSACCHARIDES

*Plant Gums.*—Continuing their studies on damson gum, Hirst & Jones (55) have isolated the following substances from among the products of hydrolysis of the whole gum, methylated by the thallous hydroxide method: 2,3,5-trimethyl *l*-arabinose (8 parts), 2,3-dimethyl *l*-arabinose (4 parts), 2,4,6-trimethyl *d*-galactose (3 parts), 2,4-dimethyl *d*-galactose (3 parts), 4?-methyl *d*-galactose (1 part), 2-methyl *d*-galactose (1 part), 2,3,4-trimethyl *d*-glucuronic acid (2 parts), 2,3-dimethyl *d*-glucuronic acid (2 parts), together with unidentified derivatives of *d*-mannose and *d*-xylose.

Previous work on the hydrolysis of methylated degraded damson gum prepared by autohydrolysis (56) had resulted in the isolation of trimethyl xylopyranose (3 to 4 per cent), 2,3,4,6-tetramethyl galactose (1 part), 2,3,4-trimethyl galactose (1 part), 2,4,6-trimethyl galactose (1 part), 2,4-dimethyl galactose (1 part), 2,3,4-trimethyl glucuronic acid (1 part), 2,3-dimethyl glucuronic acid (1 part), and a partly methylated mannose. Since 2,3,4,6-tetramethyl galactose and 2,3,4-trimethyl galactose do not appear in the hydrolysis products of the methylated whole gum, it is concluded that the *l*-arabinose molecules, which are probably all furanose in form, are joined to those particular *d*-galactose residues which yield tetramethyl- and 2,3,4-trimethyl-galactopyranose on hydrolysis of methylated degraded damson gum. During the preparation of this substance by autohydrolysis all the *l*-arabinose is removed. From the fact that 2,3,5-trimethyl and 2,3-dimethyl *l*-arabinose are produced on the hydrolysis of the methylated gum in the ratio of two to one, it follows that there must be two side

chains on each repeating unit which consist of arabinose only, one side chain consisting of a single *l*-arabofuranose residue, and the other of two such molecules linked through $C_1$ and $C_5$ with the reducing group of the disaccharide united glycosidically to the main chain. The points of attachment are clearly on galactose residues at $C_3$ and $C_6$. These facts may be represented diagrammatically as

$$[A, 1\,.\,.\,]_4\,.\,.\,. \qquad [A, 1\,.\,.\,.\,5A, 1\,.\,]_4\,.\,.\,.$$

The other terminal groups are clearly xylopyranose and *d*-glucopyruronic acid.

$$[Glu.\ 1\,.\,.\,]_2\,.\,.\,. \qquad [X, 1\,.\,.\,]\underset{trace}{.\,.\,.}$$

The molecular proportions of 2,3,4-trimethyl and 2,3-dimethyl glucuronic acid are seen to be constant for both the methylated whole gum and the degraded material, so that the above residues are evidently not united to the arabinose units; the monomethyl galactoses, however, were absent from the hydrolysis products of methylated degraded gum, from which it appears likely that the *l*-arabofuranose fragments are attached to these particular residues. The repeating unit of damson gum contains arabinose, galactose, glucuronic acid, and mannose in the molecular proportions of 3:2:1:1. Below are summarised the known facts about the composition of the resistant core of the molecule:

$$.\,.\,.\,[3, Ga, 1\,.\,]_3\,.\,.\,. \qquad .\,.\,.\,[3, \underset{\substack{6\\ \vdots}}{Ga}, 1\,.\,]_3\,.\,.\,. \qquad .\,.\,.\,[3, \underset{\substack{2\\ \vdots}}{\overset{\substack{\vdots\\ 6}}{Ga}}, 1\,.\,]_1\,.\,.\,.$$

$$.\,.\,.\,[4, \underset{\substack{3\\ \vdots}}{\overset{\substack{\vdots\\ 6}}{Ga}}, 1\,.\,]_1\,.\,.\,. \qquad .\,.\,.\,[4, Glu, 1\,.\,]_2\,.\,.\,. \qquad [\underset{\substack{2\\ \vdots}}{\overset{?}{?M}}, 1\,.\,]_4\,.\,.\,.$$

White (57) has reported the results of the methanolysis of methylated mesquite gum (from *Prosopio juliflora*). It emerges that the uronic acid which is present as a methoxy uronic acid in the original polysaccharide is a terminal unit (isolated as 2,3,4-trimethyl methyl-*d*-glucuronoside) as are some of the *l*-arabinose residues (isolated as 2,3,5-trimethyl arabinose). The resemblance to damson gum ends at this point, however, for the isolation of 3,5-dimethyl methyl-*l*-arabinosides shows that some of the arabinose units are linked through the 1,2 positions. The branched chain nature of this plant gum is shown further by the isolation of 2,4-dimethyl *d*-galactose, indicating that the galactose units are pyranose and triply linked at 1, 3, and 6. These known facts are summarised below:

$$[\mathrm{A}, 1\,.\,]_1 \ldots \qquad [\mathrm{Glu}, 1\,.\,]_1 \ldots \qquad \ldots [1, \mathrm{A}, 2\,.\,]_3 \ldots$$

$$\ldots [3, \underset{\substack{6 \\ \vdots}}{\mathrm{Ga}}, 1\,.\,]_2 \ldots$$

A = *l*-arabofuranose, Glu = *d*-glucuronic acid, Ga = *d*-galactopyranose.

James & Smith (58) have shown that the material named gum tragacanth, which exudes from shrubs of the genus Astragalus, is a complex mixture of a polyuronide (tragacanthic acid), a galacto-araban, and a glycosidic substance probably of steroid character. Methylated tragacanthic acid methyl ester gave on methanolysis 2,3,4-trimethyl $\alpha$-methyl-*l*-fucoside, 2,3,4-trimethyl methyl-*d*-xyloside, 3,4-dimethyl methylxylosides, 2,3-dimethyl methyl-galacturonoside, and a monomethyl methylgalacturonoside methyl ester. The branched chain of the repeating unit is therefore terminated by *l*-fucopyranose and *d*-xylopyranose, and the *d*-galacturonic acid residues are believed to be pyranose also and linked through $C_1$ and $C_4$ as in pectic acid. The highly branched nature of this gum is evident, but it differs from the other plant gums previously mentioned in containing galacturonic acid not glucuronic acid, and no acid residues appear as terminal groups. In connection with this research some derivatives of *d*- and *l*-fucose were prepared (59).

The neutral polysaccharide associated with the tragacanthic

acid was separated after methylation of the whole gum (60). In the products of methanolysis 2,3,5-trimethyl methyl-*l*-arabinosides, 2,3-dimethyl methyl-*l*-arabinoside, $\beta$-methyl-*l*-arabopyranoside, and dimethyl methylgalactosides were found. From the high negative rotation of the methylated polysaccharide and the ease of hydrolysis, the arabinose units are most probably furanose in form. A most interesting feature which emphasises the branched chain character of the material, if the possibility of incomplete methylation be discounted, is the isolation of $\beta$-methylarabinoside, since it follows that the units giving rise to this substance must be quadruply linked in the repeating unit. There is but little resemblance, therefore, between this galactoaraban and the araban associated with pectic acid in pectic materials (61, 62).

*Slippery elm mucilage.*—Further study of the mucilage from the bark of *Ulmus fulva* (slippery elm) by Gill, Hirst & Jones (63), in which it had been shown previously (64) that the aldobionic acid 2-*d*-galacturonosido-*l*-rhamnose, identical with that isolated from flax seed mucilage (65), was a component, has resulted in the isolation of a protein-free methylated polysaccharide. Methanolysis, followed by hydrolysis, gave 2,3-dimethyl *d*-galacturonic acid (4 parts), tetramethyl galactopyranose (2 parts), 2,4,6-trimethyl galactose (1 part), 2,3,6-trimethyl galactose (1 part), 3,4-dimethyl rhamnose (2 parts), 4-methyl rhamnose (2 parts), and a trace of 2,3,4-trimethyl galacturonic acid. From these results the following residues linked together as shown below are the building units of slippery elm mucilage, although the precise arrangement in this case as in the other polysaccharides so far discussed must be a matter for further research:

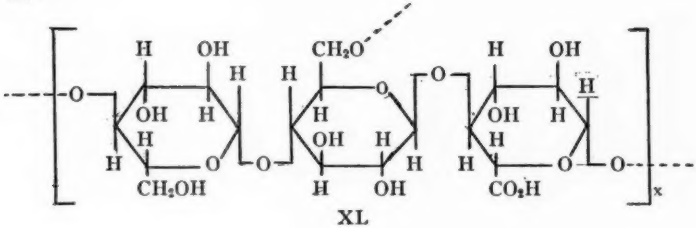
$$[\text{Ga}, 1]_2 \ldots \qquad \ldots [4, \text{Ga}, 1]_2 \ldots \qquad \ldots [3, \text{Ga}, 1]_2 \ldots$$

$$\ldots [4, \text{Gau}, 1]_4 \ldots \qquad \ldots [2, \text{R}, 1]_2 \ldots \qquad \begin{matrix}\searrow \\ \nearrow\end{matrix}\!\!\left[\begin{matrix}2\\3\end{matrix}\text{R}, 1\right]_2 \ldots$$

Ga = *d*-galactose residue; R = *l*-rhamnose residue; Gau = *d*-galacturonic acid residue. In this material there are 1,2-; 1,3-; and 1,4-linkages in the same molecule, and the galacturonic acid is substituted on $C_1$ and $C_4$ as in pectic acid.

*Rhizobium polysaccharide.*—The repeating unit of the capsular polysaccharide of ***Rhizobium radicicolum*** has been formulated as

(XL) by Schlüchterer & Stacey (66). This polysaccharide gives a preciptin reaction not only with Type III pneumococcus antiserum, but also with mixed antisera from other pneumococcus types.

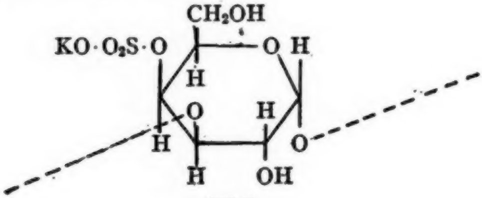


XL

*Algal polysaccharides.*—The very important fact emerges from the x-ray studies of Astbury (67) and of Palmer & Hartzog (68) that the period along the fibre axis of alginic acid is 8.7 Å as against 10.3 Å for cellulose. This is explained as due to the fact that, when the hexopyranose rings are constructed in the "chair" form using standard bonds and angles, the formation of $\beta$-linkages between the 1 and 4 positions in contiguous rings is possible in two ways. In the first of these, the two rings are inclined at an angle of about 20° with one another and a chain made up in this way is practically linear. This represents the cellulose model and the spacing agrees with the observed figure of 10.3 Å. The second alternative, however, causes the rings to be inclined at an angle of about 90°, and the result is a shortening of the period along the fibre axis to a value of 8.7 Å for each disaccharide residue, in agreement with the value found experimentally. The alginic acid structure therefore appears to be much more buckled than that of cellulose, which may have some bearing on its special properties. Similar fibre periods have been found for the galacturonic acid residues in pectin.

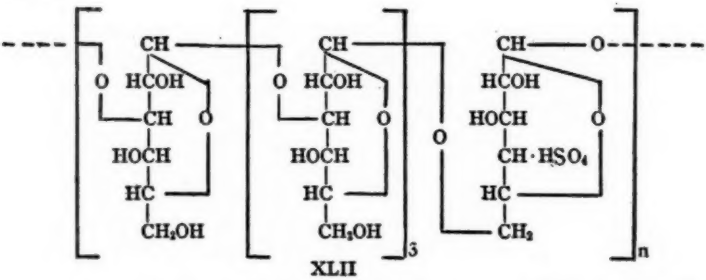
The polysaccharide extracted by hot water from the red marine alga *Gigartina stellata* Batt. has been found (69) to contain a large proportion of galactose residues, isolated on hydrolysis of the methylated polysaccharide as crystalline 2,6-dimethyl $\beta$-*d*-galactose identical with the synthetic material (70). An arrangement (XLI) of the galactopyranose residues in a way similar to that already suggested for the *Chondrus crispus* polysaccharides

(71) has been proposed, based on this evidence and on the stability of the sulphate group to alkali, a fact which appears to exclude the possibility of the presence of the sulphate group in such a position that either 3,6-anhydro or ethylene oxide rings could be produced (20, 22, 23).



XLI

Barry & Dillon (72) have isolated a galactan sulphuric ester from *Dilsea edulis* (Stackhouse) for which a tentative structure of the repeating unit has been advanced on the basis of oxidation experiments with periodic acid and treatment with phenylhydrazine (XLII). It will be seen that the fifth galactose residue is esterified by sulphuric acid on $C_4$, a position chosen on the grounds of its sluggish hydrolysis with alkali which seems to exclude the alternative possibility of linkage on $C_4$ and the sulphate residue on $C_6$.



XLII

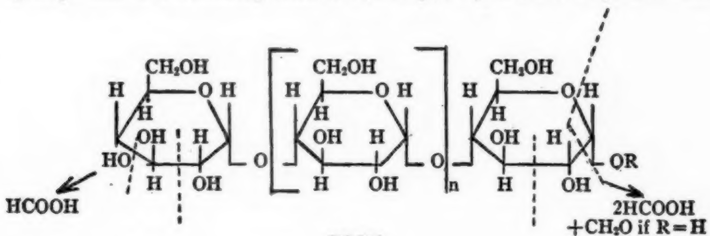
The formulation of agar suggested by Jones & Peat (73) and which requires a sulphur content of 1.8 per cent is not supported by the analyses of agars isolated from *Gelidium latifolium* which contains 0.36 per cent of sulphur (74) or from *Gracilaria confervoides* (0.43 per cent) and *Gelidium crinale* (0.47 per cent) (75). It is clear that further progress in the determination of the constitution of agar must await the results of experiments on speci-

mens of known history. This point has also been emphasised by Tseng (76) in a useful article on the terminology of seaweed colloids.

## Analytical Methods

The following summary of progress in analytical methods has been included because the progress of the subject depends to a great extent on the development of new methods of analysis and separation.

The technique of oxidation with periodic acid has been developed in a remarkable way by Hirst *et al.* (77) for the determination of end groups in polysaccharides. The method depends on the fact that in a polysaccharide built up on the pattern of starch and cellulose (XLIII) formic acid is derived only from the terminal groups and the reducing residues (if any). By the use of potassium




XLIII

chloride and sodium periodate which prevents over-oxidation owing to the low concentration of periodate ion present, conditions have been found for liberating formic acid quantitatively from the terminal sugar residues, provided no 1,6-linkages are present, in which case each unit gives rise to one molecule of formic acid. The excess of periodate is destroyed by the addition of ethylene glycol, and the formic acid estimated directly by titration or by other means. The importance of this advance in technique is obvious when it is realised that an end group determination is possible in about eight days on half a gram of material in the starch series. The results agree excellently with those obtained in five instances by the more laborious methylation technique and assay of the tetramethyl glucopyranose produced on hydrolysis. Cellulose, after making allowance for the formic acid produced by the reducing end of the chain, gives a minimum value of at least a thousand glucopyranose units per chain. The result for inulin is a chain length of twenty-five fructofuranose units as against the

previous value of thirty units (78) obtained by the methylation method which in any case is liable to give a lower value for tetramethyl fructofuranose and a consequently higher value for the chain length owing to losses due to the formation of furfural derivatives. A value of twelve units per repeating unit has been obtained for glycogens from horse muscle, rabbit liver, *Mytilus edulis*, human muscle, rabbit muscle (fasted), and *Ascaris lumbricoides*, the same value having previously been obtained for the first three by the methylation method (79, 80).

The method has been applied to gum arabic in which it is shown that the repeating unit of mol. wt. 1220 contains two end groups, in support of Smith's formulation (81), and the formula suggested by White for the ε-galactan of larch wood (82) is supported in that there are three end groups per repeating unit of mol. wt. 1104.

Bell (83) has developed a method for analysing mixtures of tetra-, tri-, and di-methyl glucoses such as are obtained in the end group assays of methylated polysaccharides, involving partition between chloroform and the aqueous solution held in a rigid silica-gel column (84). In this way he has achieved a quantitative separation of 2,3,4,6-tetramethyl glucose from 2,3,6-trimethyl glucose, and a separation of the dimethyl glucoses from the latter has also been carried out. About one gram of methylated glycogen suffices for an end group determination in this way in which the possibility of demethylation by contact with methanolic hydrogen chloride during glycoside formation is avoided since the sugars themselves are separated.

Chromatographic separations have found many applications. Hixon *et al.* (85) have shown that fluorescence chromatography can be applied to the separation of the di-, tri- and tetra-methyl glucoses using a fibrous alumina column. Wolfrom and his associates (86, 87) using "Magnesol," a synthetic hydrated magnesium acid silicate, have separated from cane juice and black strap molasses *meso*-inositol and mannitol from molasses, using the acetylated mixtures in benzene solution and using the brush method with alkaline potassium permanganate as indicator. Certain commercial clays have been used to separate mixtures of free sugars with success (88) by development with hydrophilic solvents of low molecular weight and using streak reagents, and the first

eight carbon ketose, *d*-gluco-*l*-tagato-octose, prepared by the diazomethane method from heptaacetyl *d*-gluco-*d*-guloheptonyl chloride has been purified using a "Magnesol" column (89). The chromatographic separation of synthetic octaacetyl cellobiose has already been mentioned (50).

Coleman *et al.* (90) have determined the point of linkage of disaccharides by methylation, hydrolysis and esterification of the exposed hydroxyl groups using the azobenzene-4-carboxylates. The esters so obtained are separated by chromatography on a silica column from benzene-alcohol.

A useful gravimetric method for the detection of traces and for the estimation of xylose in mixtures has been described (91) which depends on the formation of the relatively insoluble xylose dibenzylidene dimethyl acetal.

The valuable benziminoazole method of Link for characterising sugars has been extended to *d*-ribose, *d*-digitoxose and *l*-fucose (92), and Wolfrom & Karabinos (93) have described the preparation of many diethyl mercaptal acetates with characteristic melting points and rotations.

The formation of furfural and methylfurfural from pentoses and methyl pentoses has been put on a more strictly quantitative basis by Hughes & Acree (94) and a noteworthy advance in the determination of methyl pentoses has been made by Nicolet & Shinn (95) who used periodate oxidation and estimation of the acetaldehyde liberated.

A method for the separation of glucose from fructose in polysaccharide hydrolysates has been described by Bell (96) which depends on the fact that diacetone glucose, being furanose, is readily hydrolysed at room temperature by dilute mineral acid, whereas $\beta$-diacetone fructose which has a pyranose structure is not. Solvent extraction can therefore be used to separate the fructose portion.

Somogyi (97) has devised a copper reagent which by the use of a phosphate buffer is sufficiently alkaline to allow the microdetermination of maltose and other comparatively slowly reducing sugars. Browne (98) has described a method for the determination of maltose in the presence of glucose which depends on the fact that the optical rotation of glucose can be reduced to zero by the addition of sodium bisulphite, whereas the rotation of maltose

and dextrins is but slightly affected. The same author (99) had earlier provided evidence for the formation of bisulphite compounds of aldoses in solution.

A reduction method using a modified Benedict's reagent at 25° has been employed (100) for the estimation of 5-ketogluconic acid which is not interfered with by 2-ketogluconic acid, glucose or galactose.

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# THE CHEMISTRY AND METABOLISM OF THE LIPIDS[1]

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Because other chapters deal with waxes, steroids, antioxidants, choline, and fat-soluble vitamins, this review has been restricted by omission of those contributions which would be more appropriately considered elsewhere. On the other hand it has seemed desirable to include publications dated before the period under review but not available to the previous reviewers. In addition, the reader is referred to the reports of the Literature Review Committee (1, 2) of the American Oil Chemists' Society for newly recorded physical and chemical characteristics and analyses of fats and oils, and for technical information of limited interest to biochemists. A new book, ***Chemical and Physical Properties of Fatty Acids***, by Markley (3) is of interest.

## CHEMISTRY

*Determination of lipids.*—Studies on methods for technical control reflect a desire for rapid procedures. For lipids in fish, hydrochloric acid digestion (4) or rapid drying at 100° for 15 min. and then to constant weight at 150° (5), was used as pretreatment for solvent extraction, with results agreeing well with methods which are standard but more tedious and time consuming. The determination of fat and sugar simultaneously depended on alcohol extraction and separation of fat from sugar in the dried extract by use of ether (6). The Babcock butterfat test was applied, in a modified form, to the determination of fat in dried eggs (7).

Fat extraction methods for certain biochemical materials utilized innovations. Fowweather & Anderson (8) mixed feces with plaster of Paris before extraction, in order to allow determinations of both split and unsplit fat. The technic minimized hydrolysis and other destructive changes. Frampton & Giles (9) designed an apparatus for extraction of oil seeds under reduced pressure whereby the effects of prolonged heating were minimized. A new Soxhlet type laboratory oil extractor (10) has a capacity of 25 to 30 lbs. of

[1] The period covered in this review is from September, 1945 to October, 1946.

raw material; thus large samples of fat may be obtained for research.

Some analytical methods were designed to handle limited amounts of sample. The principle of determining free fatty acids, gravimetrically or volumetrically, after saponifying the sample was utilized to determine the amount of lipids in biological fluids and tissues (11, 12) and in gelatin (13). In flaxseed breeding work, where meager samples were available, the seeds were ground in a Wiley mill and extracted with petroleum ether (14). A colorimetric method (15) based on the formation of a ferric hydroxamate complex was applicable to detecting very small amounts of fatty oil on tinplate, in lubricants, etc.

*Chemical and physical characteristics.*—The iodine value of lipids from biological material has been determined on samples of 10 to 100$\mu$g. by a modified Rosenmund-Kuhnhenn method (16) and by a modified Kaufmann bromine-combining method (17). The excess absorption of iodine by castor oil in rapid methods was attributed to its hydroxyl groups. Normal iodine values were obtained by blocking the hydroxyl group with the propionyl radical (18) or by acetylation (19). Accelerated Wijs and Hanus methods, respectively, were used in the tests. Iselin (20), using fatty acid determination procedures based on iodine and thiocyanate values, demonstrated that the results on wheat-germ and poppyseed oils checked those obtained by a selective oxidation technic. Powers (21), however, pointed out that the wide variations in recorded compositions of linseed oils were inherent in the calculations, for slight errors in characteristics affected considerably the apparent composition as calculated from the data.

Marcali & Rieman (22) have devised a microprocedure for the determination of saponification values of lipids which is a modification of a recent macromethod (23) in which the titration was done potentiometrically or with double indicators. The necessity for blank determination was eliminated. The method was further modified by Ketchum (24) for semimicro determinations on samples too difficult to saponify by the Marcali & Rieman procedure.

According to two groups of collaborators (25, 26), the British Society of Public Analysts' method for determining the amount of unsaponifiable matter in lipids gives excessive values. The change in the method suggested by Sylvester *et al.* (27) should be an improvement. This involves chromatographic removal of contami-

nating free fatty acids from the solution of the unsaponifiable matter before evaporation of the solvents.

Chromatography was found applicable to estimation of squalene in oils (28) by a method intended for the detection of olive oil in oil mixtures. In another test (29) olive oil could not be distinguished from patauá oil by the Bellier value, i.e., the temperature at which the free fatty acids begin to crystallize from a solution. The author recorded the Bellier value of many Brazilian edible oils.

Rapid chilling procedures were submitted to the American Pharmaceutical Association for acceptance as standard technic in the determination of melting points of fats and waxes (30). The need for such standardization was evident in the discordant results obtained with fats such as cacao butter, chilled in various ways. The compilations of data on the thermal expansion of fish oils (31) and on the specific heat of vegetable oils from 0° to 280° (32) were of particular interest to engineers and oil processors.

Several observations of surface phenomena of lipids have been recorded. The spreading of surface films of mixtures of 1 *M* lecithin with 2 to 10 *M* oleic acid, on water over an area about 18 per cent less than the calculated area for each of the components agrees with theories suggesting a colloidal association of some sort (33). In the same field, Trapeznikov (34) measured the effects in hydrated fatty acid crystal-electrolyte systems. He elaborated on their polymorphism and on the equilibrium of the monolayers, and also measured the effects of thermal treatment and presence of electrolytes on melting points and on the kinetics of monolayer formation. The data of Douglas & MacKay (35) on surface tension of the slightly soluble normal fatty acids, $C_7$ to $C_{11}$, and aqueous solutions of these, support the theory of monolayers of closely packed molecules arranged with long axes perpendicular to the surface.

Filer *et al.* (36, 37) established and correlated x-ray diffraction patterns and the molecular weights and melting points of several synthetic glycerides. Crystallization into either $\beta$- or $\beta'$-phase was found dependent on the rate of crystal growth. The information on mixed glycerides was used in establishing the identity of glycerides of natural fats. It was applied to establish the existence of a symmetrical configuration for the 2-oleodistearin isolated from kokum butter. The latter was also confirmed by Lutton (38). The natural and synthetic glycerides had three closely agreeing crystalline

forms. Lutton (39) also recorded the different x-ray diffraction patterns of the crystalline forms of oleic acid melting at 13° and at 16°. Similar data by Trillat & Brenet (40) on $\beta$-chloroethyl esters of the $C_{12}$ to $C_{18}$ fatty acids were interpreted to indicate that the chains were inclined at the base at an angle of about 72°. Sidhu & Daubert (41) recorded the x-ray diffraction characteristics of $\alpha$-monoarachidin. This completed the data for the $C_{10}$ to $C_{20}$ series and showed the relationships of the spacings in this series.

*Fatty acid and glyceride composition.*—For new analytical data which characterizes fats the reader is referred to other reviews (1, 2). However, a review of the innovations in analytical methods, scientific interpretations, and new chemical information on the constituents of lipids follows.

Smith & Brown (42) demonstrated the practicability of crystallization technics in the analysis of fats. Their fractionation of the methylated menhaden oil acids by low temperature crystallization and distillation of the fractions, yielded a saturated portion contaminated with 5 per cent monounsaturated esters, a monounsaturated fraction containing small amounts of myristate, and a polyunsaturated fraction. In connection with this procedure, the chain lengths of the acids were determined by distillation of the methyl esters of the oil, substantially completely hydrogenated (43). The superiority of the above procedure over lead salt separation was confirmed by Hilditch & Riley (44) who used it for analysis of sunflower seed, sesame, and peanut oils. Hilditch and coworkers (45, 46) used the procedure in combination with the well-known spectrographic analyses for polyunsaturated acids and found that none of the eleostearic acid-containing oils tested contained nonconjugated linolenic acid. Also in connection with crystallization technic, Bailey *et al.* (47) published preliminary work on phase studies of fat-solvent systems. Such basic information should raise fractional crystallization studies of fats above an empirical basis.

Beadle's (48) review on applied ultraviolet spectrophotometry contains absorption curves of fats and individual fatty acids, before and after isomerization. The simplicity and utility of spectrophotometry for studying double bonds, determining composition of natural fats, and changes in the double bond systems are evident. Earlier methods were too tedious and not sufficiently accurate for conjugation studies. The utility of the technic was ex-

emplified in following the progress of the preparation of pure linoleic and linolenic acids (49); it was used to prove that linseed oils as commercially produced do not contain conjugated systems (50), and it was instrumental in the identification of *cis*, *cis*-9,12-linoleic and *cis*, *cis*, *cis*-9,12,15-linolenic acids in beef tallow (51). Raman spectra studies (52) of unsaturated fatty acids suggested that natural oleic, linoleic, and ricinoleic acids were all in the *cis*-form. In this work the $C_6$ and $C_8$ fractions, distilled from butter and a $C_{10}$ acid from castor oil gave Raman lines corresponding to the presence of a triple bond. All the above reports of spectrometric investigations contained data of particular interest as reference material.

A novel procedure for analysis of mixtures of geometric isomers of fatty acids was based on the observation that in addition-reactions involving the double bond the *cis*-isomer usually reacts much faster than the *trans*-form (53). When methoxymercuration was applied until one-third complete, followed by analysis with dithizone reagent, the error in composition was only 4 per cent.

Monick *et al.* (54) recorded vapor pressure equilibrium data for several fatty acids and their methyl esters at low pressure. This information is intended to serve as reference material for analysis by distillation procedures.

An attempt to fractionate the glycerides of coconut oil by liquid-liquid extraction by Fish *et al.* (55) failed, although some preferential solubility was evident. The fractions contained six to all eight of the acids in the oil.

The question of type of fatty acid distribution in glycerides was a part of several analytical reports. Lard and tallow analyses, by Riemenschneider *et al.* (56) suggested that the fatty acid patterns of the glycerides in the pig and cow were of random character. The percentages of trisaturated (14.7 per cent) and disaturated (45.9 per cent) glycerides of tallow were much higher than those (1.9 and 25.7 per cent, respectively) of lard. The triunsaturated glycerides (18.0 per cent) in lard were significantly higher than the literature had previously indicated. The glycerides of the seed oils of Sarda melon (57) and *Terminalia belerica* (58) approximated the rule of even fatty acid distribution, which is not met in many seed oils. Mowrah oil (59), although of about the same fatty acid composition as the *T. belerica* seed oil, deviated considerably from the rule of even distribution. Similar analyses of the glycerides of milk

fat (60) indicated that the acid distribution tended to follow more closely the "even" than the "random" hypothesis.

Some workers recorded composition variations in fats from individual sources. Anantakrishnan *et al.* (61) reported the trends in the composition changes of milk fat secreted by one cow. On the first day of lactation, colostrum fat possessed a composition intermediate in character between body fat and milk fat. A gradual decrease in oleic acid and an increase in low molecular weight acids occurred as lactation advanced. A report (62) on the variation of milk fat composition reflects changes, probably due to seasonal differences, in the feed of the cows. An analysis of human milk fat by Brown & Orians (63) indicates that it is more similar to human body fat than it is to a typical butterfat. Only small traces of acids lower than $C_{10}$ were found; the presence of $C_{10}$ to $C_{18}$ monoenoic acids was verified, and small amounts of tri- and dienoic $C_{18}$ acids were present although normal linolenic was negligible in quantity.

A project started in 1936 on the analysis of South African fish lipids (64 to 71) has continued to develop data on the distribution, composition, nature, and metabolism of lipids in fish in various seasons. In addition to the wealth of biological information which has resulted from this project, it has served as a basis for the origin and development of a large vitamin concentrate industry in South Africa (72).

Some analytical reports make particular references to the rarer fatty acids. 11-Eicosenoic acid, present as 0.05 per cent of the back fat of pigs which were fed buttermilk, is believed to have originated from milk fat (73). Archidonic constituted only a small part of the $C_{20}$ acid, and some evidence indicated the presence of di- and tri-enoic $C_{20}$ acids. Baudart (74) isolated 11,14-eicosadienoic and 8,11,14-eicosatrienoic acids from fish oils. The technic comprised fractionation by crystallization, bromination of selected fractions, refractionation, and debromination. The seed oil of *Erythrina cristogalli* contains an eicosenoic acid (75). According to Grindley (76) the presence of $C_{20}$ to $C_{24}$ saturated acids in Sudan Caesalpinioideae seed oils appears to be characteristic of the entire Leguminosae family. A newly reported natural fatty acid, 10-hydroxy-8-octadecenoic was found to be present in *Vermonia anthelmintica* seed oil to the extent of 62.4 per cent; Vidyarthi (77), the discoverer, named it vernolic acid.

An investigation (78, 79, 80) to determine why ringworm of the

scalp caused by the fungus, *Microsporon audouini*, may be endemic among children but clears up spontaneously with oncoming adolescence has led to the discovery that certain fatty acids of adult hair follicles are responsible for the fungistatic effect. The synthetic and follicular fatty acids of seven, nine, eleven, and thirteen carbon atoms were comparably effective and active in concentrations of 0.0002 to 0.001 per cent. These reports are also of extraordinary interest, because the occurrence of $C_{2n-1}$ acids in natural fats is very unusual. A report (81) on the bacteriostatic effect of fatty acids against tubercle bacilli reveals that the unsaturated acids of animal fats are more bacteriostatic than the saturated acids.

*Nonglyceride constituents.*—Chromatography, although somewhat of a failure for separation of mixed fatty acids, appears very promising for analytical and industrial separation of nonglyceride constituents. Swain (82) and Swain & McKercher (83) exhaustively studied adsorbents and solvents for separating the unsaponifiable constituents of fish liver oils. Alumina adsorbed all but 1 to 3 per cent of the original unsaponifiable material from a light petroleum solution of dogfish liver oil. The unadsorbed nonglyceride material proved to be hydrocarbon in nature. Benzene would elute the cholesterol and vitamin A from the alumina. Methanol eluted cholesterol. Following the above solvents, ethyl ether eluted chimyl alcohol. From the original adsorbate methyl chloride first perferentially removed vitamin A, followed by other constituents. On application to dogfish liver oil, concentrates of vitamin A of 25 to 250 times the potency of the original oil were possible. In somewhat similar work, Gajjar & Sreenivasaya (84) demonstrated that the major part of the vitamin A appeared in the top fifth of a column containing the adsorbed unsaponifiable material of shark-liver oil. DeSouza & Sreenivasaya (85) chromatographically removed sterols from the lipids used to study the sterol requirements of the rice moth. Spectral absorption curves of the dianilino derivative of the gossypol of cottonseed oil were prepared for reference in a rapid method of estimating gossypol (86).

Giral *et al.* (87, 88), in work on insect fats, observed that sulfur compounds appear in the fats of the family Catantopidae. According to von Fellenberg (89), of the 1.26 to 3.12 mg. per cent sulfur in rapeseed oil, all appears in the residue which separates or settles out after five months' storage. In the case of the seed oils of Cruciferae (90) the sulfur was found present as volatile organic sulfur

compounds which were responsible for the odors. Many of these volatile sulfur compounds which occur in the seeds disappear during extraction of the oil.

*Altering fats and oils.*—Heretofore, it has been known that interesterification of fatty acids occurs among glycerides under physical treatment. However until recently the information on the extent and course of the reaction was meager. Desnuelle & Naudet (91), inwhat appears to be one of the original analyses of interesterification, found that heating a fifty to fifty mixture of triolein and tristearin for five hours at 135°, in the presence of a suitable catalyst yielded tristearin, 30.3 per cent; distearomonoolein, 18.9; stearodiolein, 20.6; and triolein, 30.2 per cent. Unfortunately, the reaction may not have approached near enough to equilibrium to indicate a complete change to random distribution of the fatty acids among the glycerides. Correspondingly, but without the detailed analytical work, Norris & Mattil (92) carried out the reaction with tripalmitin and triolein under a stream of carbon dioxide, with alkali catalyst, and at a temperature of 225°. These more favorable conditions indicated that thermal interesterification tends to distribute the fatty acids at random among the glycerol radicals, for the residual wholly saturated triglyceride content was 14.3 per cent (12.5 per cent is the theoretical). The authors demonstrated some interesterifications between natural and hydrogenated fats and suggested that interesterification may possibly be one of the mechanisms involved in the digestion and metabolism of fats.

Ross *et al.* (93) produced $\alpha$-monostearin and $\alpha$-monomyristin by action of glycerol on methyl esters of the fatty acids and tabulated their characteristics. Feuge & Bailey (94) studied interesterification of cottonseed oil and glycerol. At temperatures below 200° and at equilibrium the proportions of free glycerol, mono-, di-, and triglycerides conformed closely to a pattern of random distribution. Removal of combined glycerol by high-temperature steam distillation left a mixture with decreased mono- and triglycerides and increased percentage of the diglycerides.

A trend to attribute flavor "reversion," i.e., development of characteristic off-flavors, in certain fats to the presence of polyunsaturated acids, is evident. Glimm & Nowack (95) isolated a factice-like material containing some dioxane ring stuctures from reverted tallow. These could only arise from the action of oxygen on two polyunsaturated acids. The tendency to flavor change is

reduced by hydrogenation (96) and by removing acetone-insoluble material from the oils after a polymerization treatment (97). However, sufficient elimination of the reversion tendency to enable production of a stable comestible fat from linseed oil was unsuccessful. A comprehensive and critical review on the subject was compiled by Bailey (98).

New and improved analytical methods are affording a more accurate means of studying the selectivity of the hydrogenation reaction. In this connection, Bailey & Fisher (99) represented the reactivities of fatty acids toward hydrogen under selective conditions by the following whole numbers: oleic, 1; isoöleic, 1; isolinoleic, 3; linoleic, 20; and linolenic, 40. The hydrogenation of linoleic acid appears to proceed in two stages with intermediate desorption of oleic acid from the catalyst, but with linolenic acid, part may proceed to oleic acid without intermediate desorption of linoleic. Hilditch (100) interpreted the above and several other reports of past years as suggesting that the ease of hydrogenation appears to be connected with the presence of a $-CH_2-$ group separating the unsaturated groups, in that selective addition of hydrogen was connected with the ready detachment of hydrogen from the central $-CH_2-$ group. A much lessened selective hydrogenation of free acids as compared to the esters was attributed to the competitive action of the strongly polar carboxyl group. Hydrogenation under selective conditions, followed by crystallization to remove saturated acids, was used by Swern *et al.* (101) to prepare oleic acid in good yields from tallow. *Cis*-9-oleic acid predominated in the product. The course of the physical changes occurring in ten common oils during hydrogenation was recorded by Palfray (102). His work with Delaplanche (103) on linseed oil was a graphic illustration of the progressive changes in fatty acid composition during the reduction. Similarly Joglekar & Jatkar (104) determined the most selective conditions for the continuous industrial hydrogenation process. According to Kentie & Nauta (105) rapeseed oil dissolved in dioxane, in the presence of Raney nickel catalyst and ammonium chloroplatinate as a promoter, was hydrogenated at 35°. No reduction of the carboxyl group occurred. Raymond & Moretti (106) used Raney nickel catalyst to dehydrogenate (desaturate) the $C_{12}$, $C_{14}$, $C_{16}$, and $C_{18}$ saturated fatty acids. The production of unstable liquids was submitted as evidence that desaturation took place.

Sheppard & Burton (107) bombarded fatty acids with $\alpha$-parti-

cles and reported that the following processes took place: dehydrogenation, decarboxylation, formation of low molecular weight hydrocarbons and water-soluble acids, carbon monoxide, and water.

*Synthesis of lipids.*—Details of several syntheses have been presented. Breusch & Keskin (108, 109) synthesized the $C_7$ to $C_{13}$, and $C_{15}$ $\alpha,\gamma$-diketo fatty acids and the $C_{10}$ to $C_{13}$ $\beta$-keto fatty acids and recorded their characteristics. Baudart synthesized some hydroxy $C_{16}$ acids (110) and a linoleic acid (111). The latter was a stereoisomer of the natural acid. Among the hydroxy acids prepared, the $\beta$-isomer of 9,10,16-trihydroxyhexadecanoic acid had characteristics agreeing with natural aleuritic acid, and the $\beta$-isomer of 16-hydroxy-7-hexadecenoic acid agreed with ambrettolic acid. Baer & Fischer (112) synthesized and recorded the physical and chemical constants of a homologous series of fourteen optically active pure *l*-,$\alpha$-monoglycerides containing the normal fatty acids from $C_2$ to $C_{18}$. They felt that the fact that most natural glycerides were asymmetric suggested the possibility that enzymatic reactions involving fat metabolism might also have a stereochemical basis.

Desnuelle & Naudet (113) emphasized the need of precautionary measures to guard against isomerization in synthesis of pure compounds by showing that some of the simple laboratory technics requiring temperatures of about 190° caused as much as 11 per cent isomerization of oleic acid.

Several higher fatty acid phosphates were synthesized by Lehninger (114) for testing as to whether these compounds were intermediates of fatty acid oxidation and for the natural synthesis of the glyceride bond.

## METABOLISM

*Need for fat in the diet.*—Although in the past the essentiality of fats has been dealt with almost exclusively from the standpoint of "essential" unsaturated fatty acids, there has been increasing interest recently in the presumably desirable, if not essential, role of triglycerides in respect to growth, reproduction, lactation, energy output or physical performance, economy of food utilization, relationship to other nutrients and certain less clearly defined phenomena, such as palatability and satiety.

Forbes *et al.* (115) demonstrated that weight gains, digestibility

of nitrogen, and retention of nitrogen and energy, in a seventy-day metabolism test with growing albino rats, varied directly as the fat content for isocaloric diets containing 2,5,10, or 30 per cent fat. In respiration experiments with mature rats (116) digestion and retention of food nitrogen were highest with a diet containing 30 per cent fat and the energy expense of utilization of isocaloric diets decreased with increasing dietary fat. Heat production diminished with increasing fat.

Deuel & Meserve (117), according to Deuel (118), demonstrated that rats showed optimum growth on an adequate diet containing 20 per cent cottonseed oil (33 per cent of caloric intake). Scheer (119), according to Deuel (118), found a 40 per cent fat diet to give maximum physical performance in rats, as measured by a swimming test.

Dugal *et al.* (120) found that rats kept at $-2°$ self-selected a diet of markedly higher fat content (50 per cent of total calories) than they did at higher temperatures. Survival of rats held at $-4°$ was substantially greater on a high-fat than on a low-fat diet. Mitchell *et al.* (121) have also shown that humans have a greater tolerance to cold when fed a high-fat diet and that under such conditions only a slight ketonuria and no hemolysis occurs.

Further studies have been conducted with the "essential" fatty acids. Jürgens *et al.* (122) reported that acrodynia produced in rats by feeding a fat-free diet was prevented or cured by linoleic acid but not by oleic, palmitic, stearic, 2-phytenic, 2,6-phytodienic, 10,13-nonadecadienoic (homolinoleic), 11,14-eicosadienoic, or 9,10-octadecadienoic acids. Hove & Harris (123) showed that $\alpha$-tocopherol exerts a sparing action on linoleate in the rat, presumably through protection of the double bonds, not only in the intestinal tract but also in the tissues. This is also true in certain insects where linoleic and linolenic acids were shown to be essential by Fraenkel & Blewett (124).

*Relative dietary values of fats.*—Aside from those related to "essential" fatty acids, several studies have been conducted to show the relative values of various fats in the diet. Deuel (125) has summarized the scientific evidence which led him to conclude that the fats in butter and margarine have essentially the same nutritional value. Henry *et al.* (126) concluded from their rat growth experiments that it is unlikely that butterfat possesses nutritive properties superior to those of other fats, or that the more saturated frac-

tion of butterfat is superior to that of the more unsaturated fraction or to more unsaturated vegetable oils. No growth differences were observed with butterfat, peanut, cottonseed, soybean and corn oils. Similar results were reported by Deuel *et al.* (127) who compared growth of rats on restricted and adequate calorie intakes and also on injection of growth hormone. No differences in nutritive value were found between butter, margarine, a commercial hydrogenated fat, or corn, cottonseed, peanut, or soybean oils. In a further attempt to explain the results of Boutwell *et al.* (128), which indicated a superior growth-promoting property of butterfat under certain experimental conditions, Deuel and co-workers (129) have shown that extraction of residual fat from the skimmed milk powder in the diet made no difference in the essentially equal growth responses to butter, margarine or cottonseed, peanut or soybean oils. Parrish *et al.* (130), attempting to clarify the discordant results of others, fed to rats the Wisconsin diets made in either their own or the Wisconsin laboratories, and found that differences in weight gains were significant in only two of six groups fed either butterfat or corn oil and these were related to food intake. Preference tests did not explain the variability.

Adequacy of margarine to meet the dietary needs for fat was indicated by Deuel's report (118) that rats fed a diet consisting only of skimmed milk powder, margarine fat, ground whole wheat and sodium chloride have progressed to the fifteenth generation without indication of an approaching deficiency. Euler *et al.* (131) presented evidence that certain margarine oils may be superior to butterfat when evaluated on the basis of reproduction and lactation.

Rapp *et al.* (132) compared tobacco seed oil with butterfat by rat growth. Spector (133) found no consistent distinction between the fatty acids of butterfat and of corn oil in their effects upon bacterial growth, and presumably upon bacterial synthesis of vitamins.

Henderson *et al.* (134) found synthetic triolein or milk fat added to an essentially fat-free basal diet containing linoleic acid, to increase growth equally well. Synthetic trilaurin fed at the same level restricted growth to that on the basal diet.

The results of rat feeding tests by Crampton & Mills (135, 136) with two mixtures of cottonseed oil and cottonseed stearin and with four different kinds of food fats (an animal-vegetable fat, an

all hydrogenated vegetable fat, a blended vegetable fat, and lard) fed in rations baked at 375° or 425°F. showed that the digestibility of fat or its deposition in the carcasses was unaffected by baking.

*Digestibility of fats* in vivo.—Further evidence has been presented to show that all food fats, with the probable exception of those few having large proportions of stearic acid, are digested in animals to essentially the same degree of completeness, as determined by coefficients of digestibility. Digestibilities reported (in per cent) were: (*a*) in human subjects, butterfat 97, margarine fat 97 (137); (*b*) in rats, tobacco seed oil 97.9, cottonseed oil 99.1, butterfat 98.2 (132), an animal-vegetable fat 97, a vegetable fat 98, a hydrogenated vegetable fat 97, lard 99 (each heated in the rations at 375° or 425°F.) (136), bland lard shortening 93, vegetable fat shortening 92.4 (138).

Further light has been thrown by Mattil & Higgins (139) on the factors influencing the digestibility of fats. They studied the digestibility of synthetic mono-oleodistearin, monostearodiolein, and two-to-one and one-to-two mixtures of triolein and tristearin in rats. Stearic acid was shown to be very unabsorbable, although better utilized when fed as mixed triglycerides than as tristearin mixed with triolein. Norris & Mattil (92) suggest that interesterification of glycerides may be involved in digestion.

Killian & Marsh (140), using human subjects in good health, found no relationship between fat content and gastric evacuation rate for foods such as bread, milk, cake, beef, egg, pie crust, French fried and Lyonnaise potatoes, in which the fats were in moderate quantities and intimately mixed in the food. French fried and Lyonnaise potatoes containing moderate amounts of fat left the stomach as rapidly as boiled potatoes, but "greasy" potatoes were evacuated less rapidly. From these studies it appeared that physical form, rather than quantity, of fat in a meal influences the evacuation of foods from the stomach. However, heating alone may have some effect as possibly suggested by the studies of Crampton & Mills (135, 136) and the earlier work of Roy (141, 142). Wikoff *et al.* (143) fed dogs triolein and linseed oil added to a complete ration but were unable to draw any general conclusions concerning the effects on intestinal elimination.

*Enzymatic digestion of fats.*—Schønheyder & Volqvartz (144, 145) studied beef pancreatic, pig pancreatic, liver, and human milk lipases with a large series of triglycerides and demonstrated

that calcium chloride promotes the action of pancreatic lipase by removal, as insoluble soaps, of the higher fatty acids which in the free form have a strong depressing effect on the enzyme. The optimum pH for pancreatic lipase increases from 7 to 8.8 with increasing numbers of carbon atoms in the fatty acids. Menezes & Banerjee (146) also showed that lower fatty acid glycerides are hydrolyzed by pancreatic lipase more rapidly than higher fatty acid glycerides. The digestibility of certain vegetable oils as altered by refining, was also studied. They reported that carotene, calciferol, and cholesterol, often removed during refining, accelerate the digestion of peanut and coconut oils by pancreatic lipase. Lowering of the rate of hydrolysis by developing rancidity suggested that loss of natural antioxidants may be responsible. Of the antioxidants tested, $\alpha$- and $\beta$-naphthol had no effect, hydroquinone had very little effect, resorcinol had an inhibitory effect, and Kamola dye caused considerable acceleration of lipase action in coconut oil and to a lesser degree in peanut oil.

*Absorption of fats.*—Frazer (147) presented a comprehensive and critical review of experimental findings in regard to intestinal absorption of triglyceride fats. He feels that experimental evidence favors his partition theory more than the lipolytic hypothesis of Verzár & McDougall (148).

Cooke *et al.* (149) studied the absorption of fat by 120 humans with various intestinal disturbances. Patients with diarrhea, anemia, and gastrointestinal disorders (except for those with steatorrhea) absorbed 91 to 99 per cent of a 50 gm. daily feeding of fat. In idiopathic steatorrhea absorption was much lower. Absorption was also abnormal in tropical sprue, pancreatitis, and following certain kinds of gastrointestinal surgery. Malabsorption was not attributed to more rapid passage through the intestines. Wollaeger *et al.* (150) also reported impaired fat absorption following gastrointestinal surgery.

Evidence that homogenization may influence absorption of fat in unweaned calves was presented by Bate *et al.* (151). In young calves fed skimmed milk and soybean oil separately, an alopecia was observed which could be prevented or cured by homogenizing the oil in skimmed milk or by feeding hay and grain. Frazer (152) presented histological evidence that choline seems to influence passage of fat through the intestinal mucosa. Vonk (153) suggested that gastric juices, even of invertebrates, may contain bile acids.

*Fat tolerance.*—Tolerance for fat in the diet has still not been scientifically defined. Consolazio & Forbes (154) vividly described the undesirable physical and mental effects of feeding pemmican (71 per cent calories from beef fat, 2 per cent from carbohydrate) as the sole diet to a group of men. They point out that the palatability as well as quantity of fat in a diet may influence results. Tuft & Tumen (155) believe some individuals have an intolerance to fat even at ordinary levels.

One of the possible hazards of parenteral administration of fats is suggested by Baronofsky *et al.* (156) who demonstrated that fat emboli, resulting from intravenous injection of fat, may cause gastric and/or duodenal erosions or ulcers, possibly by rendering the areas susceptible to injury by gastric juice during a temporary anemia. Ulcers were observed within an amazingly short time in cats, dogs, and guinea pigs, but not in rabbits, after injection of 1 to 2 cc. per kg. of lipids from human breast or omentum. In rabbits, ulcers were produced by intravenous fat injection only when a histamine-beeswax mixture was injected daily, intramuscularly, but not when fat or histamine-beeswax was administered separately.

*Mobilization of fats.*—Best (157) briefly summarized the literature on transport and mobilization of fats under normal, pathological, and experimental conditions.

Williams *et al.* (158) determined the distribution of total lipid, neutral fat, cerebrosides, free cholesterol, cholesterol esters, cephalin, lecithin and sphingomyelin in new-born rats and in rats at fifteen, forty-five, and seventy days of age. On the basis of percentage of total dry body weight the total lipids approximately doubled (from 21.26 to 40.72 per cent) in seventy days. During this period the neutral fat (storage lipid) fraction increased threefold (from 11.72 to 35.5 per cent), while the essential or structural lipids (total lipid minus neutral fat) decreased from 9.54 to 5.22 per cent. Whereas the essential lipid comprised 45 per cent of the total body lipids at birth it accounted for only 24, 15, and 13 per cent of the total body lipids at fifteen, forty-five, and seventy days of age, respectively. However, on the basis of percentage of neutral fat-free dry tissue substance, the total essential lipids remained relatively constant, particularly after the fifteenth day of age. The essential lipid pattern changed significantly: phospholipids and free cholesterol decreased; cerebrosides and cholesterol esters increased. The amount of dietary fat directly affected the amount

and distribution of lipids in growing rats. Foldes & Murphy (159) studied the concentrations and ratios of total cholesterol, cholesterol esters, and phospholipid phosphorus in the plasma and blood cells of healthy young and old adults as a basis for investigations on the interrelationships of these substances in pathological conditions. Williams *et al.* (160) reported the lipid distribution of brain, heart, kidney, lung, testes, liver, thymus, spleen, and skeletal muscle of rats during growth. Essential lipid (mainly phospholipid) content increased in all tissues during growth, with increases in cephalin appearing to be most important. Goldzieher (161) presented a comprehensive review of factors influencing obesity in humans.

Nordfeldt (162) found vitamin $D_3$ to increase the amount of fat in the femur of pigs but less so when either calcium or phosphorus was given simultaneously as a supplement. It was suggested that vitamin $D_3$ and calcium or phosphorus may be antagonistic to each other in respect to fat deposition in bone. Blaxter *et al.* (163) failed to demonstrate an increase in milk and milk fat yields in dairy cattle by feeding shark-liver oil.

Thayer *et al.* (164) have reported that dianisylhexene and, to a lesser extent, diethylstilbestrol, when fed to growing chickens of either sex, caused a greater deposition of subcutaneous fat, thereby giving the birds a superior dress-out quality in three to four weeks. Excessive dosage produced lipemia, among other changes. Earlier studies of Lorenz (165) showed that subcutaneous implantation of diethylstilbestrol increased deposits of subcutaneous and muscle fat in cockerels. Insignificant effects resulted from oral administration during a four week period. However, Sykes *et al.* (166) are of the opinion that feeding diethylstilbestrol to cockerels at a level of only 1 mg. per bird per day produced a definite improvement in the market quality of the chickens. Sturkie (167) found the abdominal fat to increase in cockerels injected with dienestrol and diethylstilbestrol.

Although the subject of liver fat will be reviewed in the chapter on choline, the following reports are important in considerations of lipid metabolism. Sveinsson (168) demonstrated an influence of insulin and epinephrine on mobilization of fat in the liver and muscle of rabbits fed cocoa fat. Handler (169) believes that biotin and folic acid increase the requirement for dietary lipotropic factors (choline and inositol) in rats fed low-protein, low-fat diets;

this is not the result of a specific role in lipid metabolism, but rather through their influence on food consumption which results in a greater demand on the intestinal microorganisms, which normally provide biotin, folic acid, and inositol. Albrieux & Rodriguez (170) confirmed reports of others that fat accumulates in the liver on repeated injection of liver extracts. Najjar & Deal (171) believe that methyl groups resulting from demethylation of N′-methylnicotinamide are effective in reducing the fat concentration in livers resulting from certain dietary conditions. Under other conditions, Handler & Dubin (172) were unable to show that this compound exerts a lipotropic effect.

Some of the relationships between dietary proteins or amino acids and the concentration of lipids in liver and blood have been revealed by Beveridge *et al.* (173) and Li & Freeman (174, 175, 176). Amdur *et al.* (177) found that manganese influenced deposition of lipids in liver and bone, probably through a relationship with choline. Fishman & Artom (178) suggested that the quantity of fat as well as of choline in the diet may be important in helping to maintain the lecithin content of the liver.

*The lipids in pathological conditions.*—Foldes & Murphy (179), in studies of blood lipid distribution found significant increases in plasma cholesterol, plasma cholesterol ester, and plasma phospholipid phosphorus in hypothyroidism. In hyperthyroid patients, changes in plasma cholesterol and cholesterol esters were less consistent, but plasma phospholipid phosphorus was significantly decreased. The cell lipid values seemed to be relatively unchanged both in hypo- and hyperthyroidism. The changes in concentration of lipids and the definite changes in various lipid ratios were pointed out as being consistent with the concept of Hoffmann & Hoffmann (180) regarding the pathological chemistry of hyperthyroidism. Hoffmann *et al.* (181) reported that a high-fat diet fed to rats over an extended period had no effect on metabolic rate. However, this diet permitted only about half the increase in metabolic rate by injections of thyroxine as was obtained in controls on a normal diet.

Waugh *et al.* (182) and Dixon *et al.* (183) presented detailed case histories of patients subjected to total pancreatectomy. Fat digestion and absorption were impaired so that only about one half of the ingested fat was absorbed. This condition could be substantially improved by administration of pancreatin. The

effects of total pancreatectomy on fat accumulation in the liver and changes in the lipid distribution in the blood are discussed.

Li & Freeman (184) showed that rats exposed to benzene and maintained on a high-fat diet manifested a greater incidence of leucopenia than did controls.

*Intermediary metabolism of lipids.*—Lehninger (185), working with a system consisting of washed rat liver suspension, adenylic acid, cytochrome-*c*, magnesium ions, and inorganic phosphate, which oxidizes saturated normal fatty acids with the production of acetoacetic acid found the oxidation to proceed only with the simultaneous oxidation of a second substrate or on addition of adenosinetriphosphate. It was thus concluded that the oxidation of other fatty acid substrates, in which a free carboxyl group is necessary, supplies the energy for adenosinetriphosphate formation during the oxidative phosphorylation of adenylic acid. These studies also seem to show that phosphorylation of fatty acids by adenosinetriphosphate is necessary before oxidation. Later quantitative studies by Lehninger (186) further substantiated his view that the Krebs tricarboxylic acid cycle may have importance not only in the oxidation of carbohydrates but also in the oxidation of fats. It was also demonstrated by Lehninger (187) that rat heart muscle possesses the enzymatic system necessary for direct oxidation of higher saturated fatty acids, again through the Krebs cycle.

Breusch (188) reported finding an enzyme system in skeletal muscle, kidney, brain, heart, and liver of cats and pigeons which condenses $\beta$-keto acids with oxaloacetate to give a fatty acid of two less carbon atoms and citric acid. Breusch & Keskin (189) characterized the action of the enzyme still further, and Breusch & Tulus (190) demonstrated the ability of the enzyme to oxidize all the $\beta$-hydroxy fatty acids from $C_4$ to $C_{14}$. Zeller & Maritz (191) have reported on the oxidation of $\alpha$-hydroxy acids by rat kidney extract. Certain parts of plants, such as root nodules of legumes, were shown by Süllmann (192) to contain material which accelerates the oxidation of unsaturated fatty acids. Earlier, Attimonelli (193) showed that certain species of *Brucella* oxidized $C_2$ to $C_{18}$ fatty acids but not formic acid. Klem (194) has concluded that $\beta$-oxidation is a reversible reaction on the basis of recovery of higher fatty acids from the tissues of rats previously fed deuterium-tagged fatty acids.

When $\beta$-carboxylauric acid was fed to dogs by Bernhard & Lincke (195), it was converted by $\omega$-oxidation to $\beta$-carboxy-$\gamma$-hydroxyadipic acid, which was recovered from the urine. However, $\beta$-carboxy pelargonic acid, when fed, was found in the urine unchanged. Although the exact fate of $\beta$-carboxy pentadecylic acid in the body is not known, the urine contained appreciable quantities of succinic acid after administration of this $\beta$-carboxy acid.

In continuing their studies on fatty acid metabolism, Medes and her co-workers (196), using isotopic sodium acetate, reported that conversion to ketone bodies is a major metabolic function in rat kidney. However, in rat heart muscle, acetate was oxidized without intermediary conversion to ketones. That the higher fatty aldehydes are probably not involved in the over-all synthesis or transport of fatty acids, but may be concerned with some phase of fatty acid metabolism, was suggested by Ehrlich & Waelsch (197). Tidwell & Treadwell (198) reported some findings from their extensive studies on the influence of the preceding diet on the resultant fasting ketonemia: a low protein intake favors ketone formation whereas the fat level in the diet has little, if any, influence. Geyer *et al.* (199) believe that fat is in some way involved in the utilization of galactose because the excretion of galactose by rats fed skim milk was markedly lowered by inclusion of fat in the diet.

Schmidt *et al.* (200) reported the isolation from incubated beef pancreas of $\alpha$-glyceryl-phosphorylcholine, which they believe to be the first intermediary breakdown product of lecithin to be found in mammalian tissues. It is produced from lecithin by the action of a specific lecitho-lipase which is not identical with pancreatic lipase. Annau (201), as a result of studies of the oxidation of lecithin in rabbit liver, had earlier reported an enzyme and activator in beef liver, which in the presence of methylene blue dehydrogenated lecithin.

Two interesting papers by Flössner (202) and by Kabelitz (203) report results of feeding tests with a synthetic fat, the fatty acids of which are "odd carbon" acids and essentially saturated. Extensive studies with humans and experimental animals show the fat to be well digested, absorbed, and utilized. Acidosis was reported to be less severe in diabetics fed the synthetic fat than when fed natural fats. More recently Visscher (204) fed synthetic glycerides of undecylic acid to rats as one fourth of their diets. The

fatty acid was found to be stored to the extent of approximately 24 per cent of the depot fatty acids. Thus it acted more like the higher "even carbon" acids, which are largely stored in fat depots, rather than like the lower acids, which are usually quickly metabolized.

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# PHOSPHORUS COMPOUNDS[1]

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Because phosphorus compounds are involved in a variety of metabolic functions it is difficult to discuss them without encroaching upon subjects which are reasonably parts of apparently unrelated fields and some duplication is inevitable. Fortunately, many excellent reviews are now available and only papers not contained in the reviews are cited here except where specific reference may be necessary to avoid confusion. Particular reference is made to two previous reviews on phosphorus compounds [Green & Colowick (1) and Kalckar (2)] which are exceptionally clear and comprehensive. The usual abbreviations have been used: ATP for adenosinetriphosphate, ADP for adenosinediphosphate, AMP for myoadenylic acid, DNP for diphosphopyridine nucleotide, PNA for pentose nucleic acid, and DNA for desoxyribose nucleic acid.

*Methods.*—Determination of inorganic phosphorus in the presence of labile esters (phosphocreatine, acetylphosphate, ribose-1-phosphate, etc.) is accomplished by the reduction of the phosphomolybdate complex with ascorbic acid at pH 4 [Lowry & Lopez (3)]. Burmaster's method (4) for the microdetermination of glycerophosphates by oxidation with periodate is reported to be suitable for hydrolysates of phospholipids but is probably not of more general application (5).

Improved methods for the determination of PNA and DNA based on the fact that the nucleic acids are rendered soluble by heating with trichloracetic acid have been submitted by Schneider (6) and Schmidt & Thannhauser (7) and a comparison of the two methods has been reported by Schneider (8). Rapoport & Nelson (9) have described a method for the isolation of the acid soluble nucleotides from liver involving precipitation with mercury, separation with barium, and further separation with silver. Kerr & Seraidarian (10) described methods for the separation and estimation of free purines, nucleosides and nucleotides, together with a detailed account of a method for determining pentose in

[1] This review covers the period from November, 1944 to November, 1946.

these compounds. Albaum & Umbreit (11) reported that with the Meijbaum pentose method ribose-5-phosphate (free or combined) reached complete color development in the usual heating time of twenty minutes, while ribose-3-phosphate and free pentoses required as long as forty-five minutes. The modified pentose method proposed was capable of giving precise results and also of identifying the nature of the ribosephosphate linkage providing certain interfering substances were absent.

*New compounds, synthesis, isolation.*—Lardy & Fischer (12) have described a new synthesis of glucose-6-phosphate using diphenylchlorophosphonate as the phosphorylating agent on tetraacetylglucose. The phenyl groups are removed by catalytic reduction and the acetyl groups by saponification. Reithel (13) has synthesized $\beta$-*d*-galactose-1-phosphate. Methods for the preparation of diethylchlorophosphonate (14), other alkylchlorophosphonates (15), and catechol-chlorophosphonate (16) have been described and their use as phosphorylating agents indicated.

Reich (16) especially recommends catecholchlorophosphonate which can be prepared by the action of phosphoryl chloride on pyrocatechol and which reacts readily with hydroxyl groups in inert solvents in the presence of pyridine. The protective catechol group can then be split off easily by water. Another of the more promising agents appears to be dibenzylchlorophosphonate (17, 18, 19) which reacts readily with amines, with alcohols in the presence of pyridine, and with the sodium salts of phenols. Methods of preparation (and alkaline hydrolysis rates) of glucose-6-phosphate and glucose-3-phosphate and some of their derivatives have been reported (20). Synthesis of nucleotides and nucleosides has been reviewed by Todd (21). AMP has been obtained in good yield by phosphorylating 2,3-isopropylidene-adenosine with dibenzylchlorophosphonate with subsequent removal of the additional groups. Natural adenosine itself has not yet been synthesized but a very close approach has been made by the synthesis of 9-*d*-ribopyranosidoadenine. Natural adenosine is 9-*d*-ribofuranosidoadenine.

Phosphoglycocyamine has been synthesized by treatment of glycocyamine with phosphoryl chloride and purified as the calcium salt (22). The compound was more stable than phosphocreatine to the Fiske and Subbarow method but was completely hydrolyzed in two minutes in normal hydrochloric acid at 100°. With muscle

extract, phosphoglycocyamine, like phosphocreatine, transferred its phosphate to AMP forming "delta 7" phosphorus assumed to be ATP. In the system employed it was not possible to reverse this reaction, i.e., to form phosphoglycocyamine from glycocyamine and ATP. Phosphoguanidine was not active in the system.

Lindberg (23) has isolated 1,2-propanediolphosphate from cattle brains (where it constitutes about 5 per cent of the acid soluble phosphorus) and has synthesized the compound. Addition to kidney homogenates caused an increase in pentose. Lehninger (24) has synthesized the acyl phosphates of palmitic and octanoic acids. They are relatively unstable in aqueous solution (although more stable than acetyl phosphate). An apparently specific phosphatase, present in extracts of rat liver, rapidly removes the phosphate group.

Glyceryl phosphocholine has been isolated from tissue autolysates (25, 26) and phosphocreatine has been isolated from the testes of the carp (27).

*Phosphatases, phytase, adenosinetriphosphatase.*—The occurrence, properties, and physiological significance of the phosphatases have been adequately reviewed by Moog (28) and Roche (29). Additional methods have been described by Huggins & Talalay (30) using phenophthalein phosphate and by Bessey *et al.* (31) using *p*-nitrophenylphosphate. Patients with hepatic damage have alkaline serum phosphatase levels of from four to twenty-four units per 100 ml. (normal two to five) and this increased activity could be inhibited by sodium cyanide (32). Bodansky (33) has reported on the inhibitory effect of glycine upon certain phosphatases.

Krugelis (34) and Danielli & Catcheside (35) report that in the salivary chromosomes of *Drosophilia* there exists an alkaline phosphatase localized in those regions rich in nucleic acids, not absolutely related to the Feulgen positive bands, but closely resembling the acetocarmine preparations, i.e., there is an apparent coincidence between the sites of enzymatic and of genetic activity. Lorch (36) has devised a method for the study of phosphatases in decalcified bone. A phosphatase acting on intact phosphoproteins has been reported by Harris (37).

Phytate, which is the principal storage form of phosphorus and inositol in seeds is rachitic because of the formation of insoluble calcium salts not absorbed by the intestine. The rachitic activity

may be destroyed by phytase and thus the relationship between phytate (especially in cereals), phytase, calcium and vitamin D is of considerable nutritional importance (38 to 43) but will not be discussed further here. Of particular interest, however, is the report on the properties of the phytate-protein complexes of seeds (44).

Moog & Steinbach (45) have criticized the histological methods for adenosinetriphosphatase described by Glick & Fischer (46, 47) on the grounds of lack of specificity but the latter workers point out (48) that differentiation is possible between this triphosphatase and phosphomonoesterases when they possess a different cellular distribution. Moog & Steinbach (49) followed the appearance of adenosinetriphosphatase in the chick embryo and later (50) found it to be concentrated in the small cytoplasmic granules obtained by centrifugation of extracts. By differential centrifugation it was possible to separate the apyrase activity (any enzyme releasing inorganic phosphate from ATP) from the triphosphatase (an enzyme liberating only the terminal phosphate of ATP), the latter requiring the greater field of gravity. Schneider (51) was able to associate the adenosinetriphosphatase activity of homogenates of rat liver and kidney with the large granule (mitochrondria) fraction, relatively little being found with the nuclei. The same enzyme activity has been determined in the corpora lutea and remaining ovarian tissue of the rat during the stages of pregnancy and lactation (52, 53). There is a parallel increase in size and triphosphatase activity of the functional lutein cell such as to suggest that this enzyme may be involved in the production of progesterone. However, enzyme activity per unit weight was lower in the functonal corpora lutea than in the apparently nonfunctional corpora. Adenosinetriphosphatase is reported to be high in cancer tissues (54).

*Nucleoproteins, nucleotides.*—While no complete review of this subject is intended, certain aspects merit attention here. An excellent recent review has appeared on the chemistry of the nucleic acids and nucleotides (55). The concept that the DNA proteins were present in the nucleus while the PNA-proteins were characteristic of the cytoplasm has received further support from the analysis of the separated nuclei of rat liver and kidney homogenates by Schneider (51) in which the total DNA content of the homogenates was accounted for by that present in the nuclei. There is also some indication that not only do the DNA-proteins

differ in some detail of structure but that DNA itself may differ from tissue to tissue, as, for example, in the pneumococcus transforming substance, reviewed by McCarty (56).

A relation between the nucleoprotein (of the PNA type) and the gram positive staining reaction, which is related to certain fundamental properties of bacterial cells, has been indicated for some time but the studies have now culminated in the isolation by Henry *et al.* (57) of the nucleoprotein responsible for this reaction. It was possible to separate the PNA and the protein from the isolated nucleoprotein complex and to recombine them into a gram positive staining material. The protein portion seems to be fundamentally important in the reaction. The nucleoprotein from yeast possessing the gram positive property contained 25 per cent PNA and had sulfhydryl groups; that from *Clostridium welchii* (a strict anaerobe) contained 27 per cent PNA, 3.5 per cent DNA, and had disulfide links.

Greenstein & Chalkley (58) have reported on the properties of enzymes capable of deaminating nucleic acids as well as nucleotides and Stoner & Green (59) have studied the liver and muscle deaminases acting on nucleotides. Zittle (60) has described preparations (resembling the typical animal alkaline phosphatase) which hydrolyze both PNA and DNA but which differ in several important properties from ribonuclease.

Ribonuclease itself has been studied further. Kunitz (61) has devised a spectrophotometric method based upon the fact that ribonuclease causes a shift in the ultraviolet absorption spectrum of PNA toward the shorter wavelengths. Zittle & Reading (62) have utilized the earlier manometric method of Bain & Rusch (63). Zittle (64) also reports that mononucleotides inhibit this enzyme. Since it has been demonstrated (64 to 67) that nucleotides and nucleic acids exert both stimulatory and inhibitory effects upon several oxidative enzymes, it has been suggested (67) that one of the functions of nucleic acids and their hydrolyic products (and thus one of the functions of ribonuclease) may reside in exerting a control over the rate at which oxidative enzymes may function. The earlier indications that succinic dehydrogenase was itself a nucleoprotein which were based in part upon the inhibition with ribonuclease, have become less definite, first, because nucleotides inhibit succinic dehydrogenase (67, 68) and second, because the inhibitory action of ribonuclease was apparently due to an impurity in the latter, indicated to be proteolytic in nature (68a).

This does not prove that succinic dehydrogenase is not a nucleoprotein, but removes some of the evidence which might be cited in favor of this view.

In brain, testes, liver, and heart minces of the dog, ATP breaks down to adenosine, or adenine and pentose, rather than inosinic acid (69), correlating with the relative lack of AMP deaminases in these tissues. In rat liver, 60 per cent of the acid soluble nucleotides were determined as ADP (70).

Colowick & Price (71) have prepared an enzyme (termed ribonucleic acid phosphorylase) from rat muscle which reversably replaces the guanine in PNA with phosphate. DNA, guanosine, and guanylic acid, all of which possess the ribose-1-guanine link are not active in the system. This is, of course, of particular interest since guanine is a coenzyme of enzymatic transphosphorylation in muscle (72).

Schlenk & Waldvogel (73) report that incubation of purine nucleotides and nucleosides with tissue extracts changes the ribose group in such a manner that it no longer reacts with orcinol. Free pentoses added are not changed but adenosine and guanosine are acted upon more rapidly than AMP, adenosine-3-phosphate, DPN, or inosinic acid. These compounds are acted upon by a nucleosidase to give ribose-1-phosphate but this substance is only an intermediate in further breakdown.

*Adenosinetriphosphate.*—This compound, adenine-9-*d*-ribofuranoside-5′-triphosphate,[2] occupies such a prominent place in the energy transfer system of the body that it deserves special mention. Recent evidence (21, 75) tends to confirm the conclusion of Lohmann that the pyrophosphate is attached through the stable 5-phosphoryl group. It is still an open question whether the material having the general properties of ATP occurring in a variety of species is identical in all details of structure but at least in one organism the ATP differs (76).

One aspect of the studies on the function of ATP should be considered. The issue is clearly stated by Engelhardt (77) as follows: "The view is advanced by most of the research workers that the role of ATP as the ultimate bearer of the physiological func-

[2] ATP is obtainable so far only by isolation and a fair amount of the research workers' time is devoted to preparing it [typical recent procedure (74)]. It appears that the time is certainly ripe for the preparation of ATP on a large scale by some organization for sale to research laboratories and attempts in this direction undoubtedly would be most welcome.

tions of the cell is by no means restricted to the muscle tissue only. *But if we look for experimental evidence in favor of this statement, we find it almost completely lacking.*" While there may be some question as to the meaning of "ultimate bearer," Engelhardt [(77), p. 175] elsewhere notes that "ATP is the bearer of the chemical energy generated in the course of metabolic processes and accumulated in the form of energy-rich phosphate bonds." If these are utilized to carry out the work of the cell, they relate to function. Experimental evidence that this is indeed the case and that ATP is interposed between the energy mobilizing and the energy depleting enzyme systems of the cells will be found in papers referred to in the following sections of this review. Whether these data constitute proof of the matter is dependent upon a more subtle analysis than can here be given.

*Coenzymes.*—Pyridoxal phosphate (first prepared enzymatically from pyridoxal and ATP) has been prepared chemically in an essentially pure form (78) and the analysis of the crystalline oxime derived from it has been reported (79). The compound has one phosphorus atom per molecule of pyridoxal but the position and mode of attachment remain to be determined. The evidence available is adequate to demonstrate that the 4-formyl and the 3-phenolic hydroxyl are both free (79, 80) and from analyses and hydrolysis products, position 6 is improbable, leaving positions 1 and 5 as possibilities. In addition to its function in amino acid decarboxylation and transamination reviewed elsewhere (81) it has been shown to function in the enzymatic synthesis of tryptophane (82).

There has been considerable study of thiamine pyrophosphate in yeast. Myrback *et al.* (83, 84) showed that yeast grown with good aeration contained very little thiamine or cocarboxylase and that the thiamine activity present was due to an oxidized form, first thought to be the disulfide (85), but later shown to be thiamine disulfide phosphate (86, 87). Karrer & Visconti (88) prepared the thiamine disulfide pyrophosphate and showed it to be inactive in pyruvic acid decarboxylation. Sure (89) reported that under very acid conditions certain commercial phosphatase preparations were unable to liberate thiamine from combined forms in brewers' yeast yet were able to do so from commercial wheat embryo and suggested that thiamine may exist in the form of different combinations in these materials.

Van Wagtendonk & Wulzen (90) report the isolation of one of the guinea pig antistiffness factors in a pure form. This factor is

related to phosphorus metabolism since in its absence there is a sharp decrease in readily hydrolyzable phosphorus in the liver and kidney (91) and in the concentration of ATP, ADP, and phosphocreatine (92). Anaerobic glycolysis in tissues from deficient animals may be stimulated by ATP (93).

*Tissue analysis.*—Perhaps the most extensive studies using tissue analysis as an index of physiological state have been reported by LePage (94) in which analyses of brain, muscle, liver, kidney, and heart of the rat for ATP, ADP, AMP, phosphocreatine, and several other phosphorylated intermediates are given. In the paper mentioned, data are presented not only upon animals given a variety of treatments but also upon normal animals frozen with and without anesthesia. The latter should prove invaluable for the estimation of the actual content of certain of the phosphorylated intermediates in the normal tissue. While the data are much too extensive to be adequately summarized here, it was demonstrated that the tissues of animals in shock (produced by the Noble-Collip technique and by the tourniquet method) possess greatly elevated inorganic phosphate and lactic acid, depleted glycogen, ATP, and phosphocreatine, and show marked increases in phosphopyruvate compared with normal animals. Data are also presented on nembutal shock and on animals with metrazol convulsions. A later paper describes a similar type of study with animals subject to hemorrhage (95). The value of this type of approach is evident in the papers mentioned and while the relation of phosphorylation to shock will not be discussed further here, the following papers present data on the problem (59, 96 to 99).

Other studies using this approach are those of Albaum *et al.* (100), who show that *in vivo* inhibition of cytochrome oxidase causes ATP depletion in the tissues, those of Horvath (101) showing no gross changes in phosphate distribution in aging muscles, those of Kaplan *et al.* (102) on coma produced by alloxan, those of Horvath & Tebbe (103) showing decreases in phosphate esters, particularly phosphocreatine and ATP, during the development of vitamin C deficiency, and those of Conway & Hingerty (104) showing decreases in glucose-6-phosphate and fructose-6-phosphate after adrenalectomy.

*Relation to carbohydrate metabolism.*—Excellent detailed reviews are now available on phosphorylase (105 to 108) and recent publications (109 to 112) have not materially altered the viewpoints there presented. Hexokinase has been crystallized from yeast (113

to 116). This enzyme is protected by glucose and by insulin and requires magnesium for its activity. With glucose, 1 mg. of the pure protein transferred 4.2 mg. phosphorus per min. at 30° C., and pH 7.5. It phosphorylated *d*-fructose at 140 per cent the rate of glucose, and *d*-mannose at only 30 per cent. The enzyme from yeast, however, apparently differs from that of the animal in not requiring guanine or reduced cozymase for activity. In the latter case, it has been shown by Colowick & Price (117) that the reduced cozymase is in a bound form. In the yeast hexokinase neither compound could be demonstrated. Cori *et al.* (118) have crystallized triose phosphate dehydrogenase from rabbit muscle and report that this enzyme requires a reducing agent such as cysteine for full activity in contrast to the similar enzyme from yeast. Caputto & Dixon (119) have also crystallized the enzyme from the same source and report it to be identical with triose dehydrogenase. However, glyceraldehyde is attacked at a much slower rate. Oxidation of glyceraldehyde or phosphoglyceraldehyde both require the presence of inorganic phosphate but only in the latter case is phosphorylation observed. The enzyme is very similar in properties to the myogen B previously crystallized. Aldolase has been crystallized and found to be identical with myogen A (120). Kubowitz & Ott (121) have crystallized from human muscle the enzyme transferring the phosphorus of phosphopyruvate to ADP.

In order to obtain rapid glycolysis in homogenates or tissue extracts it is becoming more apparent that considerable fortification of these is often necessary. Hence the earlier reports of lowered glycolysis in brain tissue of poliomyelitis infected mice (122) have been subject to some question, as methods for obtaining increased glycollytic rates in this tissue have been developed (123 to 126). However, inhibition of glycolysis has now been observed upon the addition of purified virus preparations to homogenates of normal mouse brain fortified with the necessary adjuncts and since inhibition is observed with glucose-6-phosphate and fructose-6-phosphate but not with hexose diphosphate, the locus of the action is thought to be the phosphorylation leading to hexose diphosphate (127, 128). Similar results were obtained, however, upon the addition of relatively greater amounts of nonneurotropic viruses, such as influenza and tobacco mosaic.

Using erythrocyte-free washed *Plasmodium lophurae* parasites (incubated 100 min. before glucose additions) Bovarnick *et al.* (129) demonstrated that the induction period required before

glucose is readily oxidized is prevented by AMP or ATP. During the induction period itself, the ATP is evidently formed after the addition of glucose. This recovery is prevented by 0.0001 *M* atabrin.

Spiegelman *et al.* report (130, 131, 132) that the presence of relatively low concentrations of azide (at levels having little or no effect upon fermentation or respiration of yeast) markedly inhibit the synthetic processes of cell division, carbohydrate and ammonia assimilation, adaptive enzyme formation, and phosphate turnover. They suggest that the azide acts to prevent the generation of energy-rich phosphate bonds.

A cell-free preparation of *Streptococcus thermophilus* (a bacterium in which the living cell ferments lactose more rapidly than glucose) fermented lactose and glucose (but not galactose) at comparable rates when supplied with ATP (133). In the absence of ATP, glucose-1-phosphate was rapidly fermented but galactose-1-phosphate was not.

Price *et al.* (134) report that in muscle extract from rats rendered diabetic with alloxan, the hexokinase reaction shows a latent period which is abolished by insulin. This effect is believed to be due to the preponderance of an inhibitory pituitary factor since it can also be produced by adding anterior pituitary extract to muscle extract from normal rats. Adrenal cortical extract, which has no effect upon the hexokinase obtained from normal animals, greatly intensifies the action of added anterior pituitary extract. In hexokinase preparations obtained from diabetic animals the adrenal extract has a marked effect without added pituitary extract. In all cases the inhibitions may be relieved by insulin. The adrenal factor occurs in the amorphous fraction since the crystalline materials prepared from such extracts have no effect.

*Oxidative generation of energy-rich phosphate bonds.*—Knowledge of the oxidative generation of energy-rich phosphate is not yet sufficiently clarified to permit any definite conclusions as to mechanism. Particularly the question of whether the passage of electrons through the cytochrome system is capable of generating energy-rich phosphate has not yet proved experimentally demonstrable. Lipmann (135) pictures a cyclic process, presumably occurring within some portion of the molecule of the carriers involved, in which there is opportunity for inorganic phosphorus to add to a double bond of the carbonyl or imino type. The dehydrogenation of the phosphate system transforms the electron potential

without appreciable loss into phosphate bond energy which is transferred to the adenylic system thus returning the carrier to its original state. If this hypothesis could be approached experimentally it might account for the observed results. Yet there may be other processes involved. Potter (99) in a study of the conditions necessary for oxidative phosphorylation in kidney homogenates has demonstrated that the phosphorylative mechanism is extremely labile and that oxidation of succinate may proceed rapidly without a net gain in phosphate bond energy under some circumstances and result in accumulation of energy-rich phosphate under other conditions. He has devised (136) a system for measuring aerobic phosphorylation in homogenates of rat kidney, liver, brain, heart, and muscle, rabbit ovary and adrenal tissue upon the oxidation of the four carbon dicarboxy acids. These may provide the experimental approach to the problem of oxidative generation of energy-rich phosphate.

*Acetylation.*—That the acetylation of at least certain compounds in tissues involves phosphate intermediates is now well established. The pertinent information on the acetylation of sulfanilamide and choline has been discussed by Lipmann (137) in a review on acetyl phosphate. Acetylation of sulfanilamide was shown to require a simultaneous respiration; ATP would replace anaerobically the respiratory reactions in cell-free preparations. Acetyl phosphate, however, appears to be completely inactive in contrast to its activity in other reactions in bacterial preparations. While the presence of an acetyl phosphatase in animal tissues [see also Shapiro & Wertheimer (138)] complicates the studies, it is difficult to explain the almost complete lack of activity of this compound. Since acetate and ATP are effective in acetylation and it has been shown by the hydroxylamine trapping technique that acetyl phosphate is formed from acetate and ATP, and since 0.001 *M* hydroxylamine will markedly inhibit acetylation it seemed improbable that an intermediate other than acetyl phosphate was involved. Lipmann, therefore, postulates that the process is organized as an "impenetrable unit" into which acetyl phosphate does not readily enter. The presence of an "impenetrable unit" in cell-free homogenates is not an impossibility since in such homogenates, particles separable by differential centrifugation and microscopically discernible do exist and these may possess the properties associated with an impenetrable unit. It is quite probable from the studies made on isotonicity that certain of these

particles do possess membranes, or at least surface forces, restricting diffusion (51, 139). The organization of the acetyl phosphate formation (which is endergonic) within the overall acetylation reaction (which is exergonic) is an attractive hypothesis but still requires further evidence.

In addition to a sulfhydryl compound, the acetylation reaction requires a coenzyme, which is apparently a nucleotide. Reports yet available do not record activity with any of the known coenzymes. Phosphorus is present in the purified preparations and is probably associated with the coenzyme since inactivation occurs by incubation with purified intestinal phosphatase. The reaction between acetate and ATP also requires a coenzyme and while the situation is not yet entirely clear, it seems probable that the coenzyme of sulfanilamide acetylation is involved in the acetate-ATP reaction.

The acetylation of choline [reviewed by Feldberg (140) and Nachmansohn (141)] to form acetyl choline is apparently the same type of reaction as the acetylation of sulfanilamide; again acetyl phosphate is inactive, although acetate and ATP show activity; upon dialysis the activity of the preparations is lost, sulfhydryl groups are required for activation as well as a coenzyme, almost certainly identical with that required for acetylation of sulfanilamide (142). Further studies on this system are reported by Feldberg & Mann (143), Lipton (144), Torda & Wolff (145), and Nachmansohn & Berman (146), the latter demonstrating the enzyme for the first time in the optic nerve. Presumably, the same reasoning which was applied to the sulfanilamide system to explain the lack of activity of acetyl phosphate would also serve to explain its lack of activity in the acetyl choline system. In this case, however, the enzyme may be prepared from extracts of acetone dried brain; upon centrifuging a considerable clarification is achieved (144, 146) and the active material remaining appears to be homogeneous (146). Hence it is more difficult to consider an "impenetrable unit" whose action must thus be taken from forces other than those of restricted diffusion and at present somewhat obscure. These difficulties may be clarified on further study or they may force the discovery of yet another phosphorylated two-carbon compound, not acetyl phosphate.

The two types of acetylation, one involving the esterification of a hydroxyl group with a fatty acid; the other the formation of what is analogous to a peptide link, would seem to be starting

points from which fruitful studies on fat and protein synthesis might be launched.

*Fatty acid oxidation.*—The activation of fatty acid oxidation by ATP in homogenates of liver and muscle (147, 148) of the rat indicates that phosphorylated intermediates are somehow involved. The acyl phosphates of certain fatty acids have been prepared (24) but no report has appeared on their oxidation.

The oxidation of octanoic acid (with ATP) leads to intermediates of the Krebs cycle. Lehninger (149) has reported that rat liver homogenates, in the presence of magnesium, malonate and ATP, oxidize octanoic acid completely to acetoacetate. Fatty acids supplied with fumarate show a decreased yield of acetoacetate and increased Krebs cycle intermediates whose formation involves, not acetoacetate, but some precursor of acetoacetate. There is a considerable body of evidence cited by Lehninger that the precursor is a two-carbon compound, not acetyl phosphate. Somewhat similar results were obtained with heart muscle except that ATP alone was not active in stimulating fatty acid oxidation but that fumarate and ATP (or AMP) were both necessary (150), and that the preparations rapidly removed added acetoacetate. Potter (151) has prepared the system from liver and has pointed out that in some cases at least, whole cells in the homogenates may have been responsible for the oxidation. In the complete cell-free system, cytochrome-*c* and potassium ions are necessary.

*The myosin-adenosinetriphosphatase reaction.*—The properties of myosin and its complexes have been reviewed from the viewpoint of protein chemistry by Bailey (152) and its relation to adenosinetriphosphatase has been discussed by Engelhardt (77), who summarizes the evidence that the two may be identical. Singher & Meister (153) were able to prepare myosin which showed double refraction of flow but possessed no triphosphatase activity (by precipitation at pH 6) and also myosin which showed enzyme activity but which possessed no birefringence (by incubation at pH 8.5 at 37.5°). These data suggested that the enzymatic properties of myosin and its mechanical properties were more subtly related than had been supposed. Price & Cori (154) have been able to separate myosin from triphosphatase. The separated material was thirty times more active per mg. of protein than myosin. About 3 per cent of the original protein of the myosin preparation could be obtained as the more active enzyme. It was reported that when removed from the myosin the adenosinetriphosphatase was

no longer activated by calcium, but was stimulated by creatine. The enzyme could be readsorbed on myosin (prepared at pH 6 and devoid of triphosphatase activity) in the presence of creatine. When so adsorbed, calcium had an activating effect. Since water extracts of muscle contained an adenosinetriphosphatase having the same properties as that separated from myosin, it was concluded that in the muscle itself the enzyme exists partly in a free, partly in a bound, form. However, Cori (155) has later reported that he was unable to repeat certain of the experiments relating to the stimulation by creatine. With carefully purified myosin preparations, creatine had no effect upon the triphosphatase activity and no phosphocreatine was formed. Cori also questions whether the activity remaining after acetone treatment of myosin preparations is a true separation of the enzyme from the myosin. Polis & Meyerhof (156) have been able to separate crystalline myosin into fractions having higher and lower activities by precipitation procedures. The adenosinetriphosphatase was contained in a fraction of crystalline myosin which possesses slight physicochemical differences which enabled it to be separated from the bulk of the myosin.

Price reports (157) that actin, prepared free from myosin, does not have adenosinetriphosphatase activity, but the form possessing high viscosity (potassium chloride treated) loses this viscosity (and double refraction of flow) upon the addition of ATP. It may be restored to its original state with the further addition of myosin. Similar results have been reported by Mommaerts (158) in a study of the molecular size and shape of the myosin and actomyosin molecules and their interaction with ATP.

Binkley (159) has proposed a chemical-mechanical theory of muscular contraction. In the contractile phase of myosin, sulfhydryl groups and phosphorylated hydroxyamino acids condense with the release of inorganic phosphate and the formation of a thio-ether, thus shortening the myosin chain. Phosphorylative cleavage of the thio-ether by ATP to produce free sulfhydryl groups and phosphorylated hydroxyamino acids causes relaxation.

A further relationship, perhaps in essence a myosin type of reaction, is the demonstration, by more refined techniques, that the energy source for the action potential in electric discharge by *Electrophorus electricus* is the breakdown of phosphocreatine (160).

A problem considered by Engelhardt (77) as similar to that of myosin is the relation of ATP to motility in spermatozoa. In addi-

tion to the work cited by Engelhardt, Lardy *et al.* (161) have shown that loss of ATP is correlated with loss of motility, and Mann (162) reports that there is a close correlation between the activity of the sperm and its ATP. In addition Mann has demonstrated a series of the phosphorylated intermediates of glycolysis in sperm (163, 164, 165). MacLeod & Summerson (166) found high adenosinetriphosphatase activity associated with washed human sperm but found no effect of the addition of ATP on motility.

*Relation to nitrogen metabolism.*—While the presence of phosphorylated intermediates in the metabolism of carbohydrates has been apparent for many years, experimental evidence of their presence as intermediates in amino acid or protein metabolism has been lacking. If ATP were interposed between the energy yielding and the energy utilizing reactions of the cell and if it were the only agent so involved, one would certainly expect phosphorylated intermediates to be concerned with amino acid metabolism particularly since several phosphorylated amino acids occur in tissues. There is, however, some indication that this is indeed the case particularly in the work of Binkley (167) on the conversion of methionine to cysteine in which ATP (phosphoserine ?) is involved.

That phosphorylated intermediates are involved in some phases of nitrogen metabolism has recently been demonstrated. Cohen & Hayano (168, 169) have shown that the conversion of citrulline to arginine (and thus to urea) by a transimination reaction involving glutamic acid occurs in homogenates of liver and kidney when these are fortified with ATP (cytochrome-*c*, oxygen, and magnesium ions are also necessary). Liver slices are not as active as the fortified liver homogenate which is probably due to the relatively low permeability of liver to glutamic acid. In the homogenates the systems forming glutamic acid from ammonia and those forming citrulline from ornithine are not active.

A new approach to the problem of peptide bond synthesis is reported by Cohen & McGilvery (170) in a study of the formation of *p*-aminohippuric acid from glycine and *p*-aminobenzoic acid in rat liver and kidney slices. In slices, the reaction requires oxygen. This in itself is not necessarily indicative of phosphorylation but further data [unpublished (171)] show that this reaction also occurs in homogenates. With these demonstrations, the problem of the occurrence of phosphorylated intermediates and the utilization of ATP in the reactions of nitrogen metabolism is experimentally opened and further progress will probably depend upon

the development of methods for measuring this type of reaction.

*Carbon dioxide fixation.*—ATP is involved in the Wood-Werkman reaction (fixation of carbon dioxide by condensation with pyruvate) in animal tissues (172, 173). Utter & Wood (174) have studied this reaction in detail in enzyme systems obtained from pigeon liver. The ATP may act either by the phosphorylation of a cofactor necessary for the reaction (although diphosphothiamine will not replace ATP) or by direct participation in the reaction (phosphopyruvate will not replace ATP).

Because there is not a strict correlation between ability to fix carbon dioxide and ability to decarboxylate added oxaloacetate, Utter and Wood suggest that a single enzyme does not mediate both processes. The carbon dioxide fixation could well occur by addition to a phosphorylated pyruvate (not necessarily the enol-phosphate compound, but one as yet unknown) rather than to pyruvate itself.

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# CARBOHYDRATE METABOLISM[1]

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## Introduction

Due to limitations of space and in accord with the established precedent of the previous reviewer (1), no attempt will be made in this article to cover exhaustively the large literature on the carbohydrate metabolism of microorganisms or higher plants, and pertinent contributions to these fields will be touched upon only incidentally.

Several reviews and texts of note have appeared. Soskin & Levine (2) have reviewed the field of carbohydrate metabolism and John (3) has contributed a text on diabetes. Review articles on diabetes (4), insulin (5), and the role of the anterior pituitary (6) are also noteworthy. Buchanan & Hastings' (7) review of the use of isotopic carbon contains much pertinent material as does that of Wood (8) on carbon dioxide fixation. Reviews of other special fields of knowledge are included herewith (9 to 15).

## Analytical Methods

Two micromethods for the determination of glycogen are worth noting. That of Van Wagtendonk *et al.* (16) depends upon development of color with iodine, whereas that of Boettiger (17) employs diphenylamine in acid solution as the reagent. Seibert & Atno (18) describe a colorimetric procedure for determination of serum polysaccharide which is based upon the use of Dische's carbazole reagent. The fact that "indigestible" carbohydrate corresponds roughly to that fraction which fails to dissolve in hot concentrated formic acid is the basis of an analytical procedure described by Guillemet & Hamel (19).

A crude assay method for amylolytic activity has been decribed by Lulla & Sreenivasaya (20). Enselme (21) has devised a procedure for the determination of succinic acid in urine, depending ultimately upon its oxidation in a Warburg apparatus by a suitable enzyme preparation.

[1] This review covers the period from October, 1945 to October, 1946.

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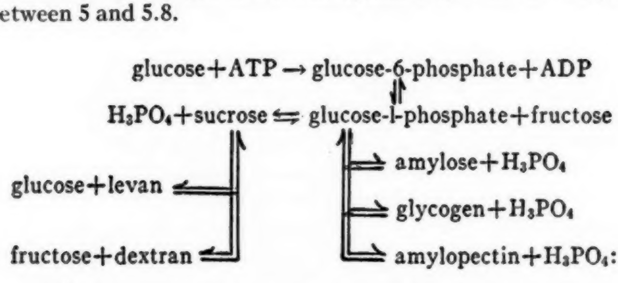
## Synthesis and Phosphorolysis of Glucosidic Linkages

The synthesis of β-methyl-*d*-glucoside from methyl alcohol and glucose catalyzed by the action of emulsin has been reported by Hérissey & Fleury (22) and the activator purified by Thoai *et al.* (23). In order to make the reaction proceed in the direction indicated, it was found necessary to operate in dioxane medium, in the virtual absence of water. Whereas this synthesis demonstrates the potential reversibility of amylolytic reactions, it appears not to be by this device that glucosidic bonds are established in nature as a rule. The general principle appears to be evolving that the energy necessary for the biological synthesis of a glucosidic bond from glucose and/or fructose fragments is derived at the expense either of another glucosidic link or of phosphoric acid acetal. From the accompanying scheme (Fig. 1) adapted from Avineri-Shapiro & Hestrin (24) it will be seen that the source of this energy may prove, in each case, to be adenosinetriphosphate. The above authors have studied levan formation from sucrose by the action of levan sucrase of *Aerobacter levanicum* and report that the yield of levan increases with rising sucrose concentration up to 3 per cent. The optimum pH of this enzyme lies between 5 and 5.8.




ATP=adenosinetriphosphate; ADP=adenosinediphosphate.

Fig. 1.—Biological origin of common polysaccharides

The reversibility of levan and dextran formation from sucrose may be inferred from experiments of Doudoroff & O'Neal (25) who employed a partially purified material from *B. subtilis*. They point out that this reversal represents a further possible procedure for biological synthesis of sucrose. Hehre (26) has studied the analogous synthesis of dextran from sucrose, catalyzed by an enzyme

from *Leuconostoc mesenteroides*. Kinetically the reaction approaches zero order when the concentration of sucrose is high, first order when sucrose concentration is low; the enzyme was not poisoned by azide, fluoride, cyanide or iodoacetate. Genghof *et al.* (27) have studied the serological properties of levans formed from sucrose and raffinose by various microorganisms. From the high degree of serological similarity of these polysaccharides they conclude that many, possibly all, bacterial levans may have serologically similar properties. Martin (28) has studied polysaccharides synthesized by soil bacteria on various media. The levan synthesized by *Azotobacter indicum* appeared to be different from that formed by *Bacillus subtilis*, both on sucrose media. On glucose medium, a dextran rich in uronic acid was formed. A hitherto unrecognized *Streptococcus viridans* isolated by Niven *et al.* (29) was found to synthesize large amounts of polysaccharide on sucrose broth. Delaporte & Belval (30) report that during the bacterial decay of previously frozen beets, both levans and dextrans are formed from sucrose.

Proehl & Day (31) have studied various polysaccharides with respect to their ability to activate potato phosphorylase. Amylopectin was extremely active whereas di- and trisaccharides were without activity. On digestion with $\beta$-amylase, amylopectin lost activity while digestion with mineral acids enhanced activity. Cyclic polysaccharides, $\alpha$- and $\beta$-Schardinger dextrins, lacking a terminal group, were inactive, but on mild acid hydrolysis they became active, presumably by ring opening. That high molecular weight was not essential for this activity was apparent from the fact that activators dialyzable through Visking could be prepared. The authors estimate that activators need not contain more than six or seven hexose units per molecule. Hidy & Day (32) in a similar study, had noted that the activation of potato phosphorylase by natural starches could be enhanced by partial acid hydrolysis of the starch. The activating ability of starch passed through a maximum at about that stage of hydrolysis at which the test with iodine became negative. In close agreement with the preceding paper, these authors suggest that seven to eight glucose residues per molecule are all that are necessary for phosphorylase activation.

The phosphorylase of waxy maize has been partially purified by Bliss & Naylor (33). Thoai *et al.* (34) find in dog intestinal

mucosa a "nonhydrolyzing phosphorylase" which phosphorylates glycogen or starch without apparent cleavage. This is probably the same process as that described by Riesser (35) and by Smits (36) for muscle pulp, an esterification of glycogen by phosphoric acid not inhibited by fluoride. Bourne & Peat (37) have separated from potato juice two enzymes, designated P and Q. The former gave, with glucose-l-phosphate, a polysaccharide exhibiting a blue iodine reaction, whereas the latter gave a product that was apparently amylopectin. It gave a red color with iodine, and studies of its enzymic hydrolysis and viscosity supported this view. The Q enzyme (38) was further shown to possess an amylolytic function which appears, in contrast to the usual amylases, to convert amylose into something resembling amylopectin.

## Amylolytic Reactions

Caldwell *et al.* (39) describe a procedure for the concentration of amylase from *Aspergillus oryzae* by repeated fractional precipitation with ammonium sulphate. In a study of amylase from the same source, as well as that derived from malt, Wallerstein *et al.* (40) have found that the active material can be precipitated by the addition of lignin at low pH, and is active when resuspended in neutral solutions. Schwimmer (41) has found that the apparent synergism of pancreatic amylase and the enzymes of *A. oryzae* with respect to the total hydrolysis of starch is due to the $\alpha$-glycosidase activity of the latter material which removes from the reaction mixture the maltose formed by the pancreatic enzyme and thus prevents polysaccharide resynthesis. A combination of pancreatic $\alpha$-amylase free of maltase and *Aspergillus* enzyme rich in maltase but devoid of $\alpha$-amylase accomplished the rapid and complete digestion of raw starch. Weinmann (42) reports that although commercial dextrins, formed from starch, initially resist digestion by taka-diastase, starch as well as maltose is digested by this enzyme, as are the dextrins formed from starch by the action of salivary ptyalin. The nutritional nitrogenous requirements of *A. oryzae* have been studied by Bindal & Sreenivasaya (43) in regard to the formation of diastase. Both the amino acid composition of the nutrient and the dimensions of the peptones supplied seemed to influence formation of the enzyme.

The amylases of various microorganisms have been studied

and compared. Bois & Savary (44), from a study of several bacterial species, find starch-splitting systems which may be classified according to whether glucose, amylotriose, or gentiobiose is formed. Kneen & Beckford (45) find that amylases of bacillary origin can be dissociated into dextrinizing and saccharifying agents. The former give rise to "Schardinger dextrins," the latter to reducing sugars. Redfern & Landis (46) have compared the liquefying and dextrinizing abilities of $\alpha$-amylases from malt, bacteria and molds.

A crystalline protein has been isolated by Balls *et al.* (47) from the press-juice of sweet potatoes, which exhibits high activity as $\beta$-amylase and only slight $\alpha$-amylase activity. Veibel *et al.* (48) report upon the glucosidase activity of *Saccharomyces fragilis* upon various natural and artificial substrates. Amylase activity of urine has been studied by Nieto (49).

Kneen & Sandstedt (50) have found in cereals a water soluble material which inhibits both the saccharifying and dextrinizing amylases of salivary and bacterial origin. Further study of this product by Militzer *et al.* (51) led to its 750-fold concentration by alcohol precipitation and adsorption on alumina. The product was nondialyzable, appeared to be a protein, was inactivated by nitrous acid and aldehyde, but proved quite stable to heat. In acid solution it was readily destroyed by various reagents. The activity of the inhibitor increases upon standing (52). The nature of the inhibition is apparently noncompetitive and it is suggested that tryptophane is a constituent of the molecule essential for its activity. Roche *et al.* (53) report that the action of pancreatic amylase upon several polysaccharides is accelerated by the presence of cysteine, alanine or combinations of these amino acids with magnesium sulfate. They find that amylases of wheat and barley are inhibited by cysteine.

## Enzymes

The revival of interest in hexokinase is manifested by two reports of the purification of this enzyme from yeast. The enzyme, which catalyzes the crucial reaction: ATP+glucose→ADP+glucose-6-phosphate, was obtained in a pure state by Berger *et al.* (54). Further crystallization from ammonium sulfate was reported not to increase the activity, 1 mg. of pure protein transferring 4.2 mg. phosphorus per minute at 30° and pH 7.5. In a simultaneous publication Kunitz & McDonald (55) report the isolation from

yeast of four crystalline proteins, one of which is apparently identical with the above hexokinase preparation. Gottschalk (56) finds that yeast hexokinase catalyzes the phosphorylation of all desmotropic forms of *d*-glucose in a solution. In contrast, of *d*-fructose, apparently only that component of the solution which is of fructofuranose structure is attacked.

Kubowitz & Ott (57) describe the partial purification of two enzymes from human striated muscle. The first catalyzes the reactions: phosphopyruvate + ADP $\leftrightarrows$ pyruvate + ATP; the second: pyruvate + reduced coenzyme $\rightleftarrows$ lactate + oxidized coenzyme.

Also from muscle, triose dehydrogenase has been crystallized by Caputto & Dixon (58) and found to be identical with triosephosphate dehydrogenase. Polis & Meyerhof (59) have concentrated adenosinetriphosphatase activity in preparations of myosin and have shown that this activity is contained only in one fraction of myosin. Price & Cori (60) have presented similar evidence of partial separation of adenosinetriphosphatase from myosin, but in a later note, Cori (61) casts doubt upon the validity of these findings. Banga (62) finds that recrystallized myosin will split ATP into ADP and inorganic phosphate.

The enzyme content of the brain of the fetal pig has been studied by Flexner & Flexner (63) who report on the activities of succinic dehydrogenase and oxidase at various stages of development. The inhibition of succinic dehydrogenase by ribonuclease has been investigated by Zittle (64). The increase in mononucleotides during inhibition was apparently insufficient to explain the adverse effect of ribonuclease upon this enzyme.

From a study of the kinetics of carbon dioxide evolution when pyruvate is treated with carboxylase and carbonic anhydrase, Conway & MacDonnell (65) conclude that the carboxylase reaction yields carbonic acid, not carbon dioxide, as an initial product.

Hogeboom (66) has separated succinic dehydrogenase from mitochondrial granules. Albaum *et al.* (67) find a difference in the rates of development, in the chick embryo, of cytochrome oxidase and succinoxidase. Schneider (68) reports that not all preparations of ribonuclease inhibit succinoxidase and from this and other evidence concludes that succinoxidase is not a ribonucleoprotein. Potter (69) has devised a method for the assay of malic dehydrogenase activity.

## Glycolysis and Respiration

Breusch & Tulus (70) find a correlation between the ability of a tissue to reduce oxaloacetate and to metabolize citrate. Lehninger's (71, 72, 73) reports reaffirm the concept that in the presence of dicarboxylic acids products derived from either fatty acid (acetate?) or glucose (pyruvate) lead to the formation of the same tricarboxylic acids. Weinhouse *et al.* (74) have demonstrated the presence of isotope in ketoglutarate isolated after shaking kidney slices with isotopic ($C^{13}$) acetate. Continuing earlier studies, Lorber *et al.* (75) have fed acetate in which both carbon atoms were labelled with $C^{13}$ and recovered glycogen in which isotope was more or less uniformly distributed. Similarly Stetten & Stetten (76) have found deuterium more or less uniformly distributed among the carbon-bound hydrogen atoms of glucose excreted in the urine of a diabetic rabbit receiving deuterium oxide. The introduction of deuterium into liver glycogen from the body water when various glycogen precursors are administered has been investigated by Stetten & Klein (77). High concentrations of isotope were found in glycogen when lactate or dihydroxyacetone served as precursor, considerably lower values when the common hexoses, glyceric acid or glyceraldehyde were given. Utter & Wood (78) find that the fixation of $C^{13}O_2$ in the presence of pyruvate and pigeon liver extract proceeds, as in bacteria, to give oxaloacetate. The carbon thus introduced is found almost entirely in the carboxyl group $\beta$ to the carbonyl, not in the $\alpha$-carboxyl (79). Mammalian kidney or amphibian liver may oxidize glucose directly without phosphorylation, according to Ruffo & Imperato (80). The stepwise degradation to triose is pictured as proceeding over gluconic acid, 2-keto-gluconic acid, pentose, etc.

Gounelle and associates (81, 82) find an increase in blood pyruvic acid after muscular exertion or convulsion. The 50 per cent lethal dose of pyruvic acid injected intraperitoneally is reported by Butturini (83) to lie in the neighborhood of 4 to 5 gm. per kg. for rats and mice. Lubin & Westerfeld (84) have investigated the formation of acetoin from acetaldehyde and pyruvate in the intact animal. They conclude, since this is characteristically a muscle reaction and since aldehyde is utilized chiefly by liver, that this is a minor metabolic pathway.

Moulder *et al.* (85) have studied the catalytic activities of

their cell-free pigeon liver extracts. The effect of concentrations of triphosphopyridine nucleotide (TPN), diphosphopyridine nucleotide (DPN) and manganese ion upon reaction rates and gas exchange are reported for: the hydration of fumarate, the oxido-reduction of malate and pyruvate, the decarboxylation of oxaloacetate, the isomerization of citrate, the oxido-reduction of isocitrate and pyruvate, the decarboxylation of oxalosuccinate, the transamination of aspartate to pyruvate, and the decarboxylation of oxaloacetate. Wiss (86), using cell-free rat liver extracts, finds that the oxidation of pyruvate, lactate or glycerophosphate is activated by addition of fumarate, succinate, malate, ketoglutarate or citrate. *l*-Histidine also appears to be essential for optimal activity. Crandall (87) has investigated the relationship between the ratio, the rate of glycogen synthesis/oxygen consumption, and the respiratory quotient of surviving rabbit liver slices and reports a positive and significant correlation.

Several studies of brain metabolism have been reported. Glycolysis by mouse brain is inhibited, according to Racker & Krimsky (88), by two factors; a diphosphonucleotidase which can be antagonized by nicotinamide, and sodium ion which is counteracted by phosphocreatine. The former inhibits utilization of triosephosphate, the latter, phosphorylation of glucose. Utter *et al.* (89) concur as to the inhibition of anaerobic glycolysis in central nervous system homogenates or extracts by diphosphonucleotidase. Relative activity of glycolysis, cord < medulla < cerebellum < cerebrum, is reminiscent of the concept of metabolic gradients. Ferris & Himwich (90) have observed similar gradient effects in a study of the loss of glycogen from the central nervous system in response to hypoglycemia. In the new-born kitten, the greatest decrease in glycogen was seen in the cord, the smallest, in the cerebral cortex. At the age of five to eight weeks, the reverse was true.

Middlesworth (91) finds that anoxia renders the intact animal less tolerant of glucose, due to retarded glycogenesis in liver and heart, but in the human D'Angelo (92) finds no such effect in simulated altitudes of ten thousand feet. Elliott & Henry (93) find that decrease in oxygen tension down to 4 mm. need not retard glycolysis of brain tissue. They describe an inhibitor of brain glycolysis present in serum (94) which can be removed by washing, and the action of which is abolished by the addition of pyruvate. Stadie & Haugaard (95), continuing their study of high oxygen pressures,

report the inactivation of succinic dehydrogenase of rat liver, kidney, and brain at a pressure of seven atmospheres. Cyanide does not prevent this inactivation, but malonate or high concentrations of succinate are protective. Reactivation of the enzyme may be effected by cysteine or reduced glutathione. Mann & Quastel (96) find respiration of brain to proceed more slowly in oxygen than in air. This is attributed to oxygen poisoning of pyruvic oxidase. Dickens (97) has studied this poisoning due to high oxygen pressure and finds that it is an irreversible injury and not due to accumulation of hydrogen peroxide. He believes pyruvic oxidase to be the enzyme system most probably inactivated, though in a second paper (98) he reports that at least five other pertinent enzymes concerned in the carbohydrate metabolism of brain tissue are sensitive to high oxygen pressure. Klein *et al.* (99) have compared the rate of transfer of levulose and glucose from blood to brain, and find that levulose is significantly more slowly transported.

Pryde (100) and Mann (101) have identified fructose as a constituent of bull semen, and the occurrence of fructolysis, with simultaneous lactate formation, is described by the latter author. Mann (102) has further studied glycolysis by spermatozoa and notes no striking differences in this process between this and other tissues. Barrón & Huggins (103) find a striking increase in citrate concentration in prostatic tumor, especially malignant neoplasm. They also report on the high concentration of aconitase in the prostate.

Kolotilova (104) has compared glycolysis in erythrocytes of various mammals and concludes that the rate-determining step lies between triosephosphate and pyruvate. Dische (105) describes aerobic glycolysis of hemolyzed avian erythrocytes and demonstrates an inhibition of this process by 0.001 *M* hydrogen cyanide and by polyvalent ions, whether anion or cation, in physiological concentrations.

The retardation in oxygen consumption by slices of heart muscle of rats in irreversible shock has been investigated by Burdette & Wilhelmi (106). They find that this retardation is not due to impaired ability of heart muscle to oxidize pyruvate. Studying the origin of cardiac glycogen, Lackey *et al.* (107) find no correlation of the quantity of this material with the level of blood sugar but obtain a positive correlation with the level of blood ketone bodies. They suggest that these may serve as a source of glycogen in myo-

cardium. Lehninger (108) reports accumulation of succinate when heart muscle is allowed to oxidize fatty acids in the presence of malonate, a finding which favors the view that products arising from fatty acids enter the tricarboxylic acid cycle.

Knodt & Petersen (109) have studied the ability of mammary gland to form and utilize citric acid. The common sugars as well as lactate and pyruvate favored citrate formation, but β-hydroxybutyrate did not do so impressively. In another paper (110) these authors report upon the effect of insulin added to the perfusate of mammary gland. Whereas the glycogen content increased, the lactose production diminished even in the presence of adequate perfusing glucose. This is of interest because it represents an isolated example of a biological disposition of glucose which, for one reason or another, is inhibited by insulin.

## Poisons

DuBois *et al.* (111) have studied the metabolic defects in rats poisoned with the rodenticide, α-naphthylthiourea. Of interest is the marked rise in blood sugar and fall in liver glycogen observed to follow the injection of 10 mg. of drug per kg. Cysteine, one gm. per kg., protected against death in these experiments. Albaum *et al.* (112) administered sodium cyanide at the level of 5 mg. per kg. to rats and observed a decrease in cytochrome oxidase activity in the brain. In this tissue decreases in glycogen, phosphocreatine, ATP, and increases in inorganic phosphate, lactate, hexose diphosphate, phosphoglycerate and phosphopyruvate were also noted. The action of phenyl mercuric nitrate upon several enzymes has been studied by Cook *et al.* (113) who find cytochrome oxidase and succinoxidase as well as lactic and glucose dehydrogenases to be inhibited. In another paper (114) the depression of yeast respiration by this poison is found to be prevented by cysteine and homocysteine, but not by cystine or methionine, in line with the accepted capacity of sulfhydryl compounds to bind heavy metals. Handler (115) has studied the metabolic defect in rabbits poisoned with cyanide, azide, malonate, fluoride, and iodoacetate. Despite the diversity of the poisons, the striking fact was the similarity of the observed response in each case. Increases in the concentrations of blood glucose and lactate, of plasma phosphate and creatine were noted, together with lesser increases in organic phosphate and creatinine. Insulin prevented the rise in glucose, but not the other

alterations. Working with rats, Handler *et al.* (116) further found that fluoride provokes a fall in muscle glycogen, which can be offset by insulin, and conclude that insulin favors glycogenesis in muscle.

Govier *et al.* (117) find that various sympathomimetic amines accelerate cocarboxylase synthesis by pigeon liver slices. The same type of amine has been shown to neutralize the effect of $\beta$-tocopherol phosphate when the latter agent is inhibiting the succinic oxidase of rat liver homogenate (118). Hepler & Simonds (119) report that uranyl nitrate, mercuric chloride and phlorhizin all cause in dogs a glucosuria which may be correlated with the degree of tubular damage but is independent of the blood sugar level or the activity of renal cortex phosphatase. Bergstermann & Stein (120) describe the poisoning of succinic dehydrogenase by quinone.

From a study of the actions of various poisons on the system: hydrogen donor-cytochrome c-cytochrome oxidase-oxygen Ames *et al.* (121) report that different metal-proteins act enzymically with various hydrogen donors; e.g., a copper enzyme with glutathione, an iron enzyme with ascorbate, and both copper and iron with succinate.

## Miscellaneous Phosphorus Compounds

Kaplan *et al.* (122) find that the fixation of inorganic phosphate into organic linkage by kidney slices is favored by the presence of fluoride even though the latter depresses oxygen uptake. Presumably under these conditions phosphate is irretrievably lost as phosphoglycerate. Horvath (123) has studied the changes in distribution of phosphorus in rat gastrocnemius muscle as a function of age and finds increases in several phosphorus fractions in the first thirty days of life. Increases thereafter are slow, and no characteristic change was detected in senescence. Dumazert *et al.* (124) have reported that dipyridyl, and methoxy-, benzyl-, 6-methoxybenzyl,- and 6-methoxydiphenyl-8-amino quinoline, as well as $\alpha,\alpha$-phenanthroline, accelerate the action of certain phosphomonesterases and pyrophosphatases. When incubated with blood (125), these same compounds caused a fall in blood glucose, a rise in "loosely combined sugar," and an initial fall in inorganic phosphate, followed by a rise.

The coupling of phosphorylation and oxidation has been discussed by Bawn & Garner (126).

Lardy & Fischer (127) describe a method for the preparation of glucose-6-phosphate. Lindberg (128) has isolated 1,2-propanediol

phosphate from cattle brain and has investigated its metabolic fate.

## Inositol

Binkley *et al.* (129) have identified inositol, together with mannitol and glucose, among the components of sugar cane and have demonstrated the presence of a phytase, an enzyme which dephosphorylates phytin, in commercial bakers' yeast. Courtois (130) has studied a phytase of vegetable origin which apparently acts specifically upon inositol hexaphosphate and products of its partial hydrolysis, but not upon glycerophosphates. Whereas the common phosphatases were without effect upon inositol hexaphosphate, they were effective on the products of the partial dephosphorylation of this substance. Inositol has been identified as a constituent of desiccated thyroid gland by Meyer (131), who reports that when perfused through the isolated rabbit heart, inositol provokes arrhythmia. Fontaine *et al.* (132) find phytic acid as a major contaminant of seed meal protein.

Stetten & Stetten (133) have prepared *meso*-inositol in which the carbon-bound hydrogen was labeled with deuterium, and after its administration to the phlorhizinized rat, have demonstrated the presence of an excess of isotope in the urinary glucose. They have thus shown the biological occurrence of the ring cleavage of inositol to glucose, the configurational possibility of which was demonstrated by Fischer (134). Chargaff & Magasanik (135) have studied the oxidation of inositol and related compounds by *Acetobacter suboxydans*. Whereas *meso*-inositol consumed a half mole of oxygen, *d*-, and *l*-inositols consumed one mole and gave rise to an $\alpha$-diketo derivative. *Epi*-inosose and *epi*-inositol consumed one-quarter and one-half mole of oxygen respectively, and from the latter reaction, a mono-keto-inositol was isolated.

Carandante (136) has studied the activity and inhibition of phytase and finds that calcium chloride with or without magnesium chloride depresses the activity of this enzyme (137).

## Alloxan Diabetes

Structural requirements for diabetogenic action have been investigated by Hidy (138). Whereas alloxan and monomethyl allozan were capable of producing diabetes mellitus in rats, dimethylalloxan, benzoyleneurea, ninhydrin, isatin, mesoxalamide, ethyl mesoxalate and sodium mesoxalate were ineffective in the dosage

tested. Laszt (139) has reported that dialuric acid is diabetogenic to rats, but the dosage is larger, repeated injection is necessary and the proportion of animals rendered diabetic is lower than when alloxan is administered. Earlier studies (140, 141) had indicated that dialuric acid was ineffective in this regard. Further analytical methods for alloxan in biological materials have been developed by Karrer *et al.* (142) based upon the fluorescence of 9-methylisoalloxazine which forms on condensation with N-methyl-*o*-phenylenediamine. Brückman (143) determines alloxan by conversion to violuric acid with hydroxylamine, and development of color with ferrous ion. He confirms the prompt disappearance, reported elsewhere, of alloxan from biological materials.

Bennett & Behrens (144) find no difference between the diabetogenic and nephrotoxic doses of alloxan. Fortunately, even with persistence of the diabetes, functional recovery from the renal injury is the rule. Other manifestations of alloxan poisoning have been described by Thomas & Emerson (145) who find basophile degeneration in the pituitary and occasional necrosis in the adrenal cortex. The doses employed by them, however, are in excess of those usually necessary to produce diabetes.

Several interesting species differences in response to alloxan have been reported. Scott *et al.* (146) find little evidence of injury to islet cells in various species of birds after administration of alloxan. Ruben & Yardumian find that in cats (147) and in rabbits (148) the enteric absorption of alloxan is sufficient to produce injury to $\beta$-cells of the islets.

Canzanelli *et al.* (149) have compared slices of brain, liver and kidney from normal rats and rats rendered diabetic with alloxan. Both normal and diabetic brain slices utilize added glucose, pyruvate, lactate, succinate or glutamate, and the rates are uninfluenced by the presence of diabetes. Amino acids other than glutamic acid apparently were not utilized. No significant differences between diabetic and normal animals were found when kidney or liver slices were studied. Laszt & Vogel (150) have compared normal and alloxan diabetic rats with respect to the rate of absorption of glucose from the lumen of an intestinal segment, and find that in diabetes this rate displays a marked increase which is correlated with the severity of the condition. This acceleration of glucose absorption is cancelled when the diabetes is controlled by insulin.

Houssay & Sara (151) report that thyroidectomy decreases and

hyperthyroidism increases the sensitivity of animals to the toxic and diabetogenic actions of alloxan. Martínez (152) confirms this effect of level of thyroid activity upon sensitivity of rats to alloxan. Martínez & Orías (153) further find that adrenalectomized rats die in the hypoglycemic phase generally within two hours after intraperitoneal injection of 10 to 50 mg. per kg. of alloxan. This dose is well tolerated by normal animals. Janes *et al.* (154) have found that the apparent improvement in certain of the manifestations of alloxan-poisoned rats after adrenalectomy may be attributed to the decrease in food intake. In paired feeding experiments it was shown that the fall in blood glucose and decrease in glucosuria could be assigned to changed dietary habit. From the same group of workers comes the report (155) that the effect of administered diethylstilbestrol upon alloxan diabetic rats is in part due to the anorexia and the associated reduction in food intake following administration of this agent.

Martínez (156) has shown an effect of diet upon alloxan sensitivity of the rat. A high fat diet increases, a high protein diet decreases this sensitivity to intraperitoneal injection of the drug.

Lowry & Hegsted (157) have investigated the thiamine requirement of the alloxan-diabetic rat and have found that it is certainly not increased, but probably decreased. They point out that the peripheral neuritis of diabetes is therefore not attributable to thiamine deficiency. The finding calls to mind the well-established relationship of thiamine requirement to carbohydrate utilization. The diabetic animal, which utilizes less glucose than the normal, might be anticipated to require less thiamine.

In an ingenious study Thorogood & Zimmermann (158) have investigated the effect of pancreatectomy upon the dog previously rendered diabetic with alloxan. A decrease in the severity of glucosuria and in the insulin requirement was noted to follow this procedure although ketosis and early death tended to occur. The explanation favored by these authors depends upon a hypothetical second pancreatic hormone whose action may be pictured as generally antagonistic to insulin and whose origin is in some cells of the pancreas other than the $\beta$-cells. This is an appealing idea in view of the relatively small insulin requirement of humans subjected to total pancreatectomy and in relation to the insulin antagonist of pituitary origin discussed below.

Kaplan *et al.* (159) have studied the coma of alloxan-diabetic

rats and find it in general similar to that of human diabetes. Their analyses indicate that the rise in plasma inorganic phosphate in this condition results in part from breakdown of organic phosphate of hepatic origin. From a study of glucose tolerance curves in rats following repeated small injections of alloxan, Shipley & Rannefeld (160) conclude that repeated small injections of this material provoke a progressive uncompensated islet injury. According to Peralta (161), the initial hyperglycemia, seen early after alloxan administration, occurs even in the adrenalectomized animal but is lacking if the hepatic nerves are divided. In the experiments of Gaarenstroom & de Jongh (162) alloxan caused marked glucosuria with but slight hyperglycemia in rats. Hypophysectomy caused disappearance of glucosuria but had no effect on the blood sugar level.

## Chemistry of Insulin

Sanger (163) finds that, assuming a molecular weight of 12,000, the insulin molecule has six free primary amino groups. Of these two are due to glycine, two to phenylalanine, and two to the $\epsilon$-amino group of lysine. These results suggest that, per 12,000 units of weight, there are four open chains in each molecule, two terminated by glycine, two by phenylalanine. Waugh (164) considers the nature of heat precipitation of insulin in acid solution and finds that fibrils initially formed subsequently aggregate into spherites. De Barbieri & Spiga (165) have reinvestigated the relation of the zinc content of insulin to its activity and find no significant correlation. According to Gijon (166) insulin inhibits the oxidation of resorcinol by alkaline iodate solution. Lens (167) has applied the technique, based on the phase rule, of the analysis of the composition of a solution in equilibrium with excess solid to studies of the purity of insulin.

## Function of Insulin

Probably the contribution to the literature of carbohydrate metabolism that has provoked most discussion during the past year is the description, by Price, Cori & Colowick (168), of the principle in anterior pituitary extract which inhibits hexokinase of muscle or brain. The implications of this discovery have been expanded in Cori's Harvey Lecture (169). There is a material present in high concentration in the anterior pituitary gland that may be shown *in vitro* to inhibit the action of hexokinase in its role of

catalyzing the phosphorylation of glucose to glucose-6-phosphate at the expense of ATP. Insulin, which apparently is without effect upon hexokinase itself, effectively blocks this inhibition, and certain, but not all, preparations of adrenocortical material prolong it. Since most of the important biological fates of glucose are pictured as proceeding by way of an initial hexokinase-catalyzed phosphorylation, it might be predicted that, were hexokinase unduly inhibited, either by excess of the pituitary material or by lack of insulin, glucose would not be effectively utilized. Conversely, if the physiological inhibition of hexokinase is obliterated, as by an excess of insulin, glucose might then be phosphorylated excessively rapidly and all products derived from glucose-6-phosphate formed at excessive rate. This general picture finds support in the observation of de Duve & Hers (170) that the administration of glucose and insulin to rabbits results in an increase in hexose phosphates of muscle. It is furthermore in accord with many observations on the intact animal. Stetten & Klein have shown that in rabbits (171) as well as in rats (172) the rate of fatty acid formation from the ingredients of a high carbohydrate diet is far below normal in the diabetic animal. Further, in the rabbit it could be shown that the rate of hepatic lipogenesis was tremendously increased by administration of insulin. Whereas the diabetic rat continued to form glycogen, it did so not directly from glucose, but rather from smaller fragments. Such glycogen as appeared in the muscle of the rat and the muscle and liver of the rabbit in response to insulin was apparently formed from dietary hexose. This conception of insulin function has been summarized by Stetten (173). That many cases of human diabetes may be due to an excess of insulin antagonist has been suggested by Mirsky (174).

There are certain objections to the acceptance of the idea that the sole function of insulin is to antagonize an anterior pituitary inhibitor of hexokinase. The well-known insulin-sensitivity of the hypophysectomized animal, recently reinvestigated by Bennett & Roberts (175), cannot be explained along these lines, but might become understandable if the production of inhibitors at sites other than the pituitary gland could be demonstrated. Whereas the hexokinase of yeast was insensitive to the action of anterior pituitary inhibitor, that from brain has been shown to be sensitive (168). Yet Canzanelli *et al.* (149) find no significant differences in glucose utilization between the brains of normal and diabetic rats. The

transfer of glucose across both renal tubule and intestinal mucosa is generally believed to be associated with phosphorylation, yet these processes, in the diabetic, are not only not inhibited, but possibly enhanced. The explanation of these and other discrepancies is not at hand, but it would appear that in the intact cells of the body, hexokinase of voluntary muscle is so situated as to be affected by humoral inhibitor, whereas in many other cells, some barrier prevents this inhibition.

The diabetic animal, incapable of deriving adequate energy from sugars, depends largely upon fat as a fuel. This is reflected in the observations of Young (176), who noted a disappearance of depot fat in animals treated with diabetogenic anterior pituitary material, with a resultant increase in body specific gravity. Young also notes nitrogen retention and weight gain in his animals, with concurrent increase in content of tissue protein, findings more commonly associated with the growth-promoting fraction of anterior pituitary. In this connection, the observation of Richter *et al.* (177) that subtotally pancreatectomized rats elected to eat a diet poor in carbohydrate and rich in fat, and throve on such a diet, is significant.

The high priority of muscle for glycogen is indicated by the experiments of Swensson (178). When insulin is injected into rats or mice, a rise in muscle glycogen usually follows, but at least in these species, a fall in liver glycogen, parallel to the fall in blood glucose, is usually observed. In contrast, de Duve and collaborators (179, 180, 181) have studied the rate of disappearance of administered glucose from the blood stream of a dog under the influence of insulin. Comparison of normal and hepatectomized animals led these authors to the conclusion that the liver accounts for 70 per cent of the glucose thus utilized.

Root *et al.* (182) have studied the effects of sugars upon diabetic patients with respect to respiratory quotient, blood lactate, and blood pyruvate levels. In the untreated diabetic, administration of glucose causes but a slight rise in these values whereas levulose causes a more marked rise, suggesting that the phosphorylating mechanism for the latter sugar is different from that required for glucose utilization. Möllerström (183) also finds this difference, in that fructose was reported to be utilized more efficiently by the diabetic not in ketosis. Ingle & Sheppard (184) find that insulin exerts an adverse effect upon survival time of eviscerated rats, re-

lated to the increase in the rate at which glucose is removed from the blood stream. Delay *et al.* (185) and Mayer-Gross & Walker (186) describe changes in composition of body fluids in insulin-shocked humans. Landauer and collaborators (187, 188) have described and studied a defect induced in chick embryos by the action of insulin, notably "rumplessness." The entire and active insulin molecule is apparently necessary for this effect.

Nath & Brahmachari (189) report the inactivation of insulin by intermediary products of fat metabolism, especially ketoacids, and Goldberg & Jefferies (190, 191) find that nicotinyltaurine and sulfanilylacetic acid potentiate insulin. Foglia (192) has studied the effect of subtotal pancreatectomy upon the weights of the remaining organs of the rat. He has also studied the response of these animals to administered glucose (193) and reports that the ability to make liver glycogen, which is present shortly after operation, is lost in the later stages, incident probably to atrophy of the surviving islets.

Dixon *et al.* (194) report another case of total pancreatectomy in the human and again stress the mildness of the diabetes that results from this procedure. Clinical reports of hyperinsulinism are found in the papers of Isaacs (195) and Conn *et al.* (196). In a case of insulin-resistant diabetes studied by Felder (197) the presence of a humoral insulin antagonist was demonstrable and this finding again suggests the possible usefulness of the insulin tolerance test in distinguishing between diabetes due to primary hypoinsulinism and diabetes due to an excess of insulin antagonist, possibly inhibitor of hexokinase.

Miscellaneous relationships of insulin to other body systems include the following: A study of the urinary albumin from diabetic urine by Harvier *et al.* (198). A suggestion that stimulant doses of x-ray are of benefit in the treatment of diabetes [Soler *et al.* (199)]. The finding of McFarland *et al.* (200) that the visual threshold rises during insulin-induced hypoglycemia. A description of "skin diabetes" by Urbach (201), and a demonstration of the sharp rise in skin sugar in diabetes (202). The failure to find any increase in the rate of disappearance of isopropanol from the blood when insulin was administered [Lehman (203)].

## Insulin and Epinephrine

Sveinsson (204) reports on the effect of epinephrine and insulin upon the composition of the rabbit liver previously rendered fatty

by an excessively high fat diet. Administration of insulin, with or without glucose, resulted in increase in liver fat content, doubtless due to the stimulus to lipogenesis afforded by insulin (172). Epinephrine likewise resulted in a rise in liver fat and decrease in liver glycogen. Weissberger (205) has studied the distribution of radioactive phosphorus in the blood of guinea pigs after administration of radioactive phosphate as influenced by insulin and epinephrine. Both agents resulted in a reduction in quantity of blood inorganic phosphorus and a rise in muscle hexosemonophosphate in the intact animal. The former effect was attributed to insulin, in both cases, the latter to epinephrine, as insulin does not provoke a rise in hexosemonophosphate in the muscle of the adrenalectomized animal (206). No change in specific activity of any of the blood fractions was noted in response to either agent, possibly a result of the intracellular nature of the hexokinase reaction. The survival time of the isolated respiratory center of the rat was studied by Hiestand & Nelson (207) who report that epinephrine increases this value even when, due to previous inanition, a hypoglycemia results. Insulin results in a subnormal survival time, possibly related to the hypoglycemia.

Pazzo (208) observes an increase in blood lactate when epinephrine is injected into patients suffering from liver disease. This undoubtedly results from the known capacity of epinephrine to favor the breakdown of muscle glycogen to lactate, a reaction which becomes dominant when liver glycogen is not available.

## Adrenal Cortex

Venning *et al.* (209) describe an assay method for the carbohydrate-active adrenocortical steroids in urine and elsewhere, which depends upon the increase in liver glycogen of mice given small quantities of glucose and the material to be tested. Tested by this technique, 11-dehydro-17-hydroxycorticosterone is three times as active as 11-dehydrocorticosterone. Another method described by Eggleston *et al.* (210) depends upon the repeated administration of the test material to previously adrenalectomized mice and the determination of how well the liver glycogen is maintained. Dorfman *et al.* (211) have applied this method to the analysis of various adrenocortical preparations and have evaluated the range of error. The results (212) agree well with those of Venning *et al.* (209). Eversole (213) finds but little effect upon the survival time of adrenalectomized rats on carbohydrate-poor diets incident

to the administration of desoxycorticosterone acetate. Segaloff (214) on the other hand reports that after subcutaneous implantation of pellets of desoxycorticosterone acetate to similar animals on similar diets both survival and growth indicate effective substitution therapy. Doetsch (215) has been able to show that phosphorylation of glycogen by muscle as well as by liver of adrenalectomized animals proceeds more slowly than in normal controls and Montigel (216) reports similar observations. The latter author finds in addition that phosphorylation may be restored to normal rate by the addition of desoxycorticosterone.

Ingle (217) has pressed the procedure of forced feeding of rats to the point of obtaining glucosuria in the normal. He and his collaborators (218) have applied this technique to a study of the altered metabolism after pancreatectomy and after administration of adrenocortical steroids. In both conditions, the glucosuria was associated with weight loss and increases in urinary nitrogen, potassium, sodium and chloride. The "diabetes" resulting from administration of steroids proved to be insulin-resistant.

The administration of adrenotropic pituitary hormone to rats and guinea pigs was found by Sayers *et al.* (219) to result in a prompt fall in adrenal ascorbic acid and a more gradual fall in adrenal cholesterol. An excess of glycogen was deposited in the livers, suggesting that an excess of active steroid, possibly formed from cholesterol, was being released by the adrenal cortex. Abelin (220) reports a rise in adrenal cholesterol in rats after a high carbohydrate meal and finds that the cholesterol content of the adrenal gland may be caused to double by continued high carbohydrate feeding. Rats with an elevation of adrenal cholesterol produced by this means were found to store more liver glycogen after carbohydrate feeding.

## Sex Hormones

Estrogen, according to Boettiger (221), causes an increase in uterine glycogen of normal as well as ovariectomized rats. Clavert & Duval (222) report that the pigeon liver glycogen increases at the period when the follicles commence to enlarge, and that muscle glycogen increases slightly in the periods of egg-laying and hatching. Folliculin injected into male or female pigeons (223) produced a slight fall in liver glycogen and a small rise in muscle glycogen. Sendrail *et al.* (224) have found that following castration in the guinea pig, there is an increase in insular tissue of the pancreas and

a more or less permanent tendency toward hypoglycemia. Smith (225) finds that after ovariectomy in the rat there is an increase in glycogenesis during fasting and a tendency toward elevation of plasma sodium, together with cytological changes in the adrenal cortex. These findings suggest that an increased cortical secretion has occurred, possibly secondary to excess stimulus by the anterior pituitary adrenotropic factor. After subtotal pancreatectomy in rats, Foglia (226) finds that males develop diabetes more severely and more promptly than do females. Decourt (227) states that in human diabetic material, administration of estradiol benzoate or testosterone propionate results in a fall in blood glucose. The decrease in glucose tolerance seen in pregnancy has been studied by Hurwitz & Jensen (228).

## Thyroid

According to May *et al.* (229) thiourea administration results in a marked increase in the liver glycogen content of rats, an effect not offset by thyroid administration when food intake was controlled. Whereas thiouracil and other related compounds had no effect upon the cytochrome oxidase activity of thyroid tissue, thiourea had a mild inhibitory action, according to McShan *et al.* (230). No inhibition of succinic dehydrogenase was noted. Tipton *et al.* (231) find an increase in the activity of succinoxidase and cytochrome oxidase in the livers of rats fed desiccated thyroid. This increase is largely overcome by adrenalectomy.

The effect of thyroidectomy upon the diabetes incident to subtotal pancreatectomy has been studied by Houssay *et al.* (232). In thyroidectomized rats, removal of 95 per cent of the pancreas is frequently not followed by diabetes and the remaining islet tissue does not degenerate. The administration of thyroid to such animal results in the development of diabetes.

## Vitamins

The report from Merezhinskiĭ & Cherkasova (233) indicates that insulin shock lowers the vitamin C stores of the body. Conversely the vitamin C deficient guinea pig responds more violently and more promptly to insulin, with early lethal outcome if this vitamin has been completely lacking. Conceivably this observation is related to the findings of Murray & Morgan (234) that vitamin C deficient guinea pigs have slightly higher blood sugars

and slightly lower glycogen reserves six hours after feeding of glucose. Under hypoxic conditions, the deficient animals maintained blood glucose and liver glycogen levels better than did normal controls.

Wickson & Morgan (235) have failed to find the expected rise in liver glycogen in rats exposed to low oxygen tension if the animals had been on riboflavin deficient diets. After fasting, the blood glucose values in these animals were lower than in control rats, and the authors therefore conclude that normal gluconeogenesis requires the presence of riboflavin in the diet.

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# THE METABOLISM OF PROTEINS AND AMINO ACIDS[1]

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In reviewing the work of the first year following the cessation of hostilities it is perhaps appropriate that some consideration be given to the disturbances of protein metabolism which result from physical trauma and protein insufficiency.

## Protein Depletion and Regeneration

*Blood proteins.*—Rossiter (1) has described the pattern of recovery of the blood proteins of liberated Indian prisoners-of-war suffering from extreme protein insufficiency. When first studied, these patients had a normochromic macrocytic anaemia, a reduced bodyweight, and a reduced serum protein concentration; the latter was confined almost entirely to the albumin fraction, and hence there was a reduction in the albumin-globulin ratio, a slightly reduced plasma volume and, because of the low haematocrit, a significantly reduced blood volume. There was therefore a reduction in the total circulating haemoglobin and total circulating plasma protein. When these men were given a diet rich in calories, protein, and vitamins, the above factors returned to normal according to a definite pattern. The first stage, lasting up to four weeks, was characterised by a rapid rise in plasma volume to normal and the disappearance of edema. The blood volume rose steadily but at such a rate that, although the haemoglobin concentration and the haematocrit fell, the total circulating haemoglobin increased. Although the haemoglobin concentration fell, the red corpuscle concentration usually increased, since the patient's blood was rapidly becoming less macrocytic. The total circulating plasma protein increased, but because of the rapid rise in plasma volume, the plasma protein concentration changed but little. More albumin was formed than globulin.

The second stage (two to twelve weeks) was characterised by a rapid rise of both the blood volume and the total circulating plasma protein to normal. The plasma volume, which had attained normal values in the first stage, increased rapidly to values well

[1] This review covers the period from December, 1945 to November, 1946.

above normal. There was also a rapid increase in bodyweight and in total circulating haemoglobin. The total circulating albumin increased more rapidly than the globulin, but the latter did, however, reach figures in excess of normal. The third stage (eight to sixteen weeks) was marked by the transition from the second stage to normal values.

Famine edema has been produced experimentally by Keys and his co-workers (2) in thirty-four normal men who lost a quarter of their bodyweight while subsisting for six months on a European type of semi-starvation diet consisting of whole cereals, potatoes, turnips, etc., providing an average of 49 gm. protein daily. Pitting edema appeared within two months in some of the men and eventually in all but a few. The ratio of extracellular water to cellular tissue was roughly doubled. This development of edema was accompanied by only a slight decline in plasma protein concentration, averaging 0.73 gm. per 100 ml. The venous pressure was roughly 50 per cent below normal. Data from the field lend support to these indications that famine edema is not simply a result of hypoproteinaemia or of renal or cardiac failure.

Injury by heat, cold, necrotising agents, bone fracture (3) or sulphur and nitrogen mustard vesicants (4) causes a decrease in serum albumin and an increase in $\alpha$-globulin. The electrophoretic studies of Chanutin & Gjessing (4) show sharp spikes in the $\alpha$ and $\beta$ areas which have been interpreted as an indication of the appearance of abnormal proteins in the serum. Similar changes in the electrophoretic patterns are known to occur where there is inflammation or tissue destruction (5 to 9). The earliest change is the decrease in albumin which is probably due to a loss of albumin through damaged capillaries. The rise in $\alpha$-globulin and the change in appearance of the $\beta$-globulin pattern are probably due to the destruction and subsequent disintegration of tissue.

Electrophoretic studies have been made on the plasma proteins under conditions of protein depletion. Zeldis *et al.* (10) have found that chemical separation of albumin and globulin was much less complete than electrophoretic separation, the latter showing a much greater fall and slower recovery of the albumin following a low protein diet and plasmapheresis. Protein depletion in the dog has been found by Chow *et al.* (11) to reduce markedly the total circulating albumin, to reduce slightly the total $\gamma$-globulin and to leave unchanged the $\alpha$-globulin. Owing to a fall in total plasma

volume, the apparent effect of protein depletion was to increase $\alpha$-globulin concentration.

Clinical and experimental observations indicate that the liver plays a definite part in the regulation of the protein content of the plasma (12, 13). The operative procedures of laparotomy and partial hepatectomy have been found by Chanutin *et al.* (14) to produce somewhat similar effects on the plasma proteins of rats. On the second and third days after the operation the plasma fibrinogen and globulin were elevated in both series of animals while the albumin was below the normal value. This evidence is held by these workers not to support the thesis that the liver is directly involved in the fabrication of plasma proteins. Berryman *et al.* (13) have followed the blood proteins in dogs subjected to complete hepatectomy by the three-stage method. Observations were also made on normal dogs, and on dogs after procedures by which alterations of hepatic function were produced. Immediately after the complete removal of the liver there was a small loss or dilution of total protein and albumin, a marked loss of fibrinogen and euglobulin, and an increase of pseudoglobulin in the plasma. There was little evidence of loss or addition of protein to the plasma in subsequent periods up to thirty hours after removal of the liver. Munro & Avery (15) have now examined plasma from five normal dogs and three hepatectomized dogs by electrophoretic analysis without finding any marked change in the relative concentrations of the various components of the plasma.

Albanese *et al.* (16) have endeavoured to solve the apparent contradiction between the growth studies of Womack, Kemmerer & Rose (17), Tarver & Schmidt (18), and Womack & Rose (19), which showed that cystine is only capable of stimulating growth when methionine is present in suboptimal amount, and the conclusions of Madden & Whipple (12) that methionine is not an efficient substitute for cystine in the regeneration of plasma proteins of experimental hypoproteinaemic dogs. Albanese *et al.* have now found that in the absence of cystine in the diet the cystine required for the formation of blood albumin and globulin in the immature and adult rat can be derived from methionine. Hypoproteinaemia and anaemia were induced in their animals by a methionine- and cystine-deficient diet. The dietary factors which cause suboptimal growth of the immature rats do not appear to influence the blood protein levels. In general, better nitrogen re-

tention has been obtained after oral than after parenteral administration of protein digests (20). There is some evidence that haemoglobin may be used by the dog as a source of nitrogen for plasma protein regeneration (20).

Cohn and co-workers (21) have achieved fractionation of the plasma proteins by low temperature alcohol precipitation methods. The available evidence indicates that many of the antibodies are $\gamma$-globulins (22). By making use of a peptic digestion step and certain minor modifications it is possible to extract relatively pure $\gamma$-globulin from plasma protein fractions containing $\beta$-globulins as well (23, 24). Antibody activities were not destroyed under the conditions employed. A lactoglobulin possessing all the immune properties of colostrum has been isolated in high yield and is equivalent to plasma $\gamma$-globulin in producing anaphylaxis in the guinea pig. This was indistinguishable from $\beta$-lactoglobulin (25).

The greatest increment in plasma protein concentration normally occurs during the period of maximum growth (26). The albumin fraction increases rapidly in the later phases of growth. No consistent change was observed in the $\alpha$- and $\beta$-globulin fractions but the $\gamma$-globulin fraction increased rapidly during the first month after weaning and thereafter remained above the initial concentration.

The amino acid requirements for the formation of polymorphonuclear granulocytes and erythrocytes, as determined in protein-depleted rats (27, 28), fall into line with the requirements for body growth described by Rose. Folic acid is a necessary factor in the diet.

Although haemoglobin formation has a "high priority call" on available protein in the organism, the feeding to the rat of a diet containing either an inadequate amount of dietary protein (29, 30), or a qualitatively incomplete protein (31 to 35), will result in a mild to chronic anaemia. On the whole there appears to be a general parallelism between the haematopoietic value of a protein and its ability to support somatic growth.

The contradictory reports about the nutritive value of haemoglobin are apparently due to differences in the amino acid composition of haemoglobins from different species (36). For example, dog haemoglobin is deficient in methionine but not in isoleucine; human haemoglobin contains methionine but no isoleucine.

That the qualitative needs for nitrogen during growth are identical with those required by the adult during the replenishment

of the tissues after protein starvation has been indicated by some workers. More recent work (37) has shown that under such circumstances lysine is an essential constituent of the diet for the adult rat. For the regeneration of plasma protein in the dog arginine is necessary even though it is not essential for maintenance (38).

A promising technique for determining biological values, has been designed on the basis used by Cannon and co-workers for assessing the effects of blood substitutes on serum protein regeneration and weight recovery of hypoproteinamic rats (39).

*Catabolic response to injury.*—The protein depletion which follows moderate to severe injury is due essentially to two or more of five main causes: (*a*) loss of actual tissue; (*b*) loss of blood or exudate from the damaged area; (*c*) loss due to the excessive protein catabolism which normally follows injury and subsequent surgical manipulations; (*d*) loss due to infection if that be superimposed; (*e*) disuse or reflex atrophy [Cuthbertson (40, 41)].

In injuries, such as fractures, dislocations and even meniscectomies, the protein loss is due to two factors: atrophy and excessive catabolism of body protein (42 to 48). Of these two factors the excessive catabolism of protein, which reaches a maximum usually between the fourth and eighth day in injuries due to direct violence, constitutes the major cause of protein depletion. Following the peak of nitrogen loss there is a gradual decline though subsequent surgical procedures may cause further disturbance. Even after a month there may still be a slight negative nitrogen balance; but normally there is a slow merging into an anabolic phase (49). In burns all five causes generally operate to produce a very marked loss of body nitrogen (50 to 55). The available evidence (56, 43, 44, 57, 48) indicates that the excessive amounts of nitrogen (mainly as urea), sulphur (mainly as inorganic sulphate), and inorganic phosphate, which appear in the urine of patients with fractures, are derived from protein, and the magnitude of the sulphur to nitrogen ratio suggests that they are derived from muscle (43). In addition creatine and potassium are lost (57). In man the negative nitrogen balance in the first ten days after a fracture of the leg may amount to a loss of as much as some 856 gm. protein or 8 per cent of the total body protein (43). This is some three to four times the total protein content of the liver and this organ cannot, therefore, be the source of the material catabolized. Disuse atrophy, though a contributory factor, does not provide an adequate explanation (58). The increased catabolic processes are more general than local and

appear to be conditioned by a reflex mechanism which leads either to a raiding of the body protein reserves in order to supply endogenously the necessary substrate of amino acids for the reparative process, or to a mobilization of oxidizable material for the enhanced metabolism of the healing process and for the "alarm reaction" of Selye (59). The fact that there is a parallel rise in the excretion of nitrogen and sulphur, at least in fracture cases, suggests that there is not a preferential retention of thio amino acids. It has been suggested that this reflex exists in order to render the healing process independent of food supply (43). The so-called "toxic destruction of protein" in fever also may be explained in this teleological fashion.

When an animal has been depleted of its protein reserves no increase in the rate of loss of body protein occurs on fracture (60) or on production of a sterile abscess (61). More recently (62) it has been found that, in animals with fractures, the greater the proportion of protein in the diet before and after injury, the greater the nitrogen loss.

Most recent work indicates that blood loss due to the bleeding of a peptic ulcer will induce a very definite loss of nitrogen (63, 64). It has been suggested (65) that the loss of nitrogen in the urine and the high nonprotein nitrogen level in the blood are in part due to hypoxia consequent on haemorrhage. The observations by Croft & Peters on burns (55) do not bear this out.

Emerson & Binkley (66) administered intravenously to three patients the ten amino acids which are essential for the growth of the rat. A retention of nitrogen far in excess of the amount of nitrogen administered in the amino acid mixture apparently resulted in two patients with thoracic wounds and one with infective hepatitis, who were on high protein, high calorie diets. An increase in the protein content of the diet did not have a similar effect. It is suggested that this supplement of essential amino acids may have met the special needs of the damaged tissues so that the protein of the diet could then be more readily devoted to the normal anabolic needs of the body.

In young rats with fractures induced in two legs it was found that daily intraperitoneal injections of 1 mg. of anterior pituitary growth hormone, begun on the day of fracture and continued for five days thereafter, led to approximately the same decrease in nitrogen excretion, as occurred with their respective controls

(67). Other workers (68) using older animals and with only one limb broken have shown that a crude extract of the gland does not promote nitrogen retention although it prevents the loss which normally characterises similar injuries. Reports on the use of methionine or high protein diets in diminishing the loss of nitrogen which almost invariably follows severe injuries have not been corroborated in the case of patients being treated for hernia. The nitrogen loss of the patients on a low calorie (1,000 Cal.), low protein intake was not affected by the administration of 6.0 gm. methionine (69). Despite substantial increases in the intake of abundant protein rich food by patients with moderate or serious injuries, a negative nitrogen balance usually exists at the height of the catabolic period (44). A similar situation has also been reported in acute infections (70, 71).

It is considered that the ingestion of protein in adequate amounts is as effective or more effective preoperatively than corresponding increments made available in the post-traumatic phase, for a condition of nutritional imbalance may be present in a patient for a considerable time before he is submitted to operation [Varco (72)]. The operative procedures may accentuate the degree of negative balance, as the metabolisable material required for the processes of repair may necessitate a further breakdown of protein. This latter response is known to be conditioned by the nutritive state of the organism (60, 61, 62). Liver dysfunction may develop, particularly when the preceding diet has been scanty.

Although considerable progress has been made, protein digests on the whole do not as yet appear to offer an entirely satisfactory solution to the problem of successful parenteral maintenance of nitrogen equilibrium, owing to the large volumes of fluid required. In experiments to determine whether the practical advantages offered by the increased rate of infusion achieved with intravenously administered essential amino acids in their natural form (in contrast to racemic mixtures), might be offset by increased urinary loss, Silber *et al.* (73) found that there was a lower kidney threshold for *d*-amino acids. The proportions of the essential amino acids in the urines of dogs given amino acid mixtures did not equal their proportions in the mixtures infused and were not greatly altered by protein depletion. The response to an infusion of a standardised amino acid mixture might be used as a reflection of the state of protein reserves.

With vomiting as a criterion of intolerance, it was found that mixtures of natural amino acids were much better tolerated by the dog than mixtures of racemic amino acids (74). For certain of the latter mixtures, glycine improved the tolerance markedly. Of single racemic amino acids tried, methionine was the most poorly tolerated.

*Kidney.*—In an attempt to determine whether the edema of protein deficiency (edema of malnutrition) is accompanied by impairment of renal function Dicker and co-workers, (75) have studied kidney function in adult rats fed on protein-deficient vegetable diets. Although no evidence was obtained for a relation between the incidence of visible edema and the results of the clearance estimations, renal lesions were found in many rats on the protein-deficient vegetable diets. These consisted of necrosis and calcification of the broad limb of Henle, the glomeruli and other parts of the nephron showing no apparent change.

*Liver.*—Kosterlitz and co-workers (76 to 83) have examined the effects of fasting and of variations in dietary protein on the composition and structure of the liver cell. Compared with rats on an adequate stock diet, fasted rats and rats fed on diets qualitatively and quantitatively deficient in protein lost from their livers considerable amounts of protein, phospholipid and nucleic acid. Rats fed on high protein diets showed an increase in these three liver constituents. There was no change in the number of liver cell nuclei. Protein and phospholipid were lost or gained at identical rates while the changes in nucleic acid were of smaller magnitude. Indirect evidence has been adduced which suggests that only cytoplasmic ribonucleic acid and not nuclear desoxyribonucleic acid is affected. From these findings the authors conclude that the changes in liver protein which are associated with variations in dietary protein intake are caused not by gains or losses of an inert storage protein but by gains or losses of whole cytoplasm, both particulate and interparticulate matter taking part. The easily lost fraction of liver cytoplasm they have called "labile liver cytoplasm" in contrast to the "remaining liver cytoplasm." Male rats form more labile liver cytoplasm and retain it better than female rats. The amount of liver cytoplasm, as measured by the protein, phospholipid and nucleic acid contents or by the amount of nonglycogen, nonlipid liver solids, is directly proportional to the logarithm of the protein intake. Regression lines have been constructed for different

proteins and utilised for the assay of their biological values, thus reviving an earlier suggestion of Addis *et al.* (84).

The response of the liver to carbon tetrachloride injury is markedly influenced by the quantity of casein in the diet (83), even in short-term experiments. It has been found that regeneration of liver tissue proceeds equally well whether "labile" liver cytoplasm is absent, as in rats fed on a protein-free diet for three days, or is present in normal or excessive quantities, as in animals fed for three days on diets with a normal or a high percentage of casein. The structure of the newly formed cells is determined by the protein content of the diet in a manner similar to that observed in normal livers. A rather unexpected finding has been of interest: the histological changes, particularly the presence of degenerating edematous cells and the deposition of neutral lipids, were least marked in the animals fed on the protein-free diet.

Harrison & Long (85) have also used regeneration of liver protein following a forty-eight hour fast as a means of assaying the nutritional adequacy of dietary protein. By the use of their method it was shown that the addition of methionine or cystine to a diet containing an inadequate amount of casein will significantly increase the amount of liver protein regenerated after fasting. Such results confirm the importance of methionine in protein synthesis demonstrated by Croft & Peters (55) in their nitrogen metabolism experiments in burned rats.

The nucleic acid content of the livers of rats under different conditions has been determined by Davidson & Waymouth (86). On fasting, the total nucleic acid concentration rose slightly, but the content of nucleic acid per liver fell, and this fall in nucleic acid on fasting was due to loss of ribonucleic acid from the cytoplasm of the liver cells. The desoxyribonucleic acid remained constant. Of the total nucleic acid content of the liver tissue, a large proportion (at least 70 per cent) is present in the form of ribonucleic acid in the cytoplasm.

In the cytoplasm of liver cells from fed rats, masses of material which absorb ultraviolet light at a wave length of 257 m$\mu$ have been observed (87), and are considered to be identical with the phospholipid nucleoprotein particles which are known to contain ribonucleic acid. Fasting is accompanied by a decrease in the amount of cytoplasm and by the disappearance of these cytoplasmic masses.

## Special Phases of Amino Acid Metabolism

*Enzymes.*—The only enzymes which are known to attack simple intact proteins ***in vitro*** are the proteolytic ones; yet the tracer studies of Schoenheimer (88) and others demonstrate that substitutions of amino acids appear to occur and that reactions take place which involve active available groups in the protein molecule. In the light of this discrepancy it is of interest that Sizer (89) has demonstrated that both crude and highly purified mushroom tyrosinase can oxidise the tyrosine groups of such proteins as trypsin, pepsin, chymotrypsin, casein, peptone, insulin, and haemoglobin. The reaction does not destroy the biological activity of the three enzymes. Tyrosinase did not oxidise gelatin, protamine, and gramicidin, which are devoid of tyrosine. Certain proteins containing tyrosine were resistant to tyrosinase: but all the members of this group were oxidised by tyrosinase after a preliminary treatment with crystalline trypsin.

Greenstein & Leuthardt (90) have found widespread occurrence and powerful activity of dehydropeptidase in the tissues of rats, mice, rabbits, and guinea pigs, as compared with the relatively weak activity intracellularly of the peptidases for saturated chain peptides (amino peptidase, carboxypeptidase, etc.). Their work lends weight to Bergmann's (91) original view that amino acids can be catabolized when in peptide linkage.

*Amino acid identification and estimation.*—Gale (92, 93, 94) has used cell-free enzyme preparations from bacteria for the estimation of amino acids. His discovery of the amino acid decarboxylases of bacteria has opened up a field of investigation which has so far provided enzyme preparations for the quantitative estimation of natural lysine, arginine, histidine, ornithine, tyrosine, and glutamic acid. Since the reaction proceeds quantitatively and at an acid pH the carbon dioxide liberated can be measured manometrically and used to estimate the amount of the amino acid substrate. It has been possible to select organisms which can provide only one of these enzymes or, in other cases, to treat nonviable preparations in such a way that only one enzyme survives treatment.

The partition chromatogram technique for the separation of amino acids was first used by Gordon *et al.* (95) and later improved by Consden and co-workers (96). The principle of the method is the separation in space on a filter paper due to the different solubilities of each amino acid in the water which is invariably held

in the cellulose fibres of ordinary filter paper and in a solvent, nonmiscible with water, which is allowed to creep slowly along the filter paper past a spot which contains the amino acid mixture. The amino acids are drawn along at definite speeds behind the moving solvent and thus arrange themselves in a characteristic order. The two dimensional (two solvent) method makes possible the identification of the amino acids and peptides.

The question of the constancy of position of the spots when pure amino acid mixtures or protein hydrolysates are separated on the chromatogram has been dealt with fully (96). Dent (97) has now applied the technique to identify amino acids in various body fluids including urine. He has also used the simpler one dimensional (one solvent) technique. By trial and error with synthetic mixtures it has been possible to match closely in size and strength the spots on the chromatogram obtained from the biological fluid under test.

A study of a cystinuric patient suggested that the one dimensional strip method would be of diagnostic value. Serum and ascitic fluid can be run on the two-dimensional chromatogram provided the proteins are precipitated by ten times the volume of alcohol followed by final concentration on a water bath to an amino nitrogen content of 50 mg. per 100 ml. Bile pigments do not appear to interfere and even two dimensional chromatograms have been possible on 50 c. mm. of urine from rats with acute necrosis of liver (97)

The separation of substances of different mobility by ionophoresis in a slab of silica jelly offers another useful technique for the study of the amino acid composition of biological material (98, 99).

At present microbiological methods have been advanced for the assay of some seventeen of the amino acids, including all those essential for growth of the rat. With hydrolysates of pure proteins estimations are more consistent and accurate probably to within $\pm 10$ per cent (100), but when dealing with foodstuffs containing a variety of substances, the interpretation of the results is less certain owing to interfering substances. By adoption of standard techniques improvement could be effected (101).

*Biological value of proteins.*—Mitchell & Block (102) have made some interesting comparisons between the percentage deviation in essential amino acid content together with tyrosine and cystine and the biological value of food proteins, using whole egg pro-

tein as a standard of reference. For each food protein the amino acid with the greatest percentage deficit from the corresponding content of whole egg protein was taken as the amino acid limiting the nutritive efficiency of that particular protein, due consideration being given to the reciprocal relation between cystine and methionine in anabolism. There was a high degree of correlation ($r = -0.86$) between the percentage deficits of the limiting essential amino acids, with reference to whole egg protein, and the biological values of the proteins, as estimated by the nitrogen metabolism method. The regression equation for the prediction of biological value (y) from the percentage deficit (x) of the limiting amino acid was $y = 102 - 0.634x$. These workers suggest that the food proteins which have lower biological values than would be expected from their amino acid composition may be found to show an improvement in biological value on heating, while food proteins, the biological values and chemical composition of which show reasonable agreement, may sometimes show a decrease in biological value on heating.

The observations of Melnick *et al.* (103) indicate that the rate at which amino acids, in particular methionine, are released during digestion partly determines the biological value of a protein. Henry & Kon (104) have pointed out that in order that two proteins may exert a supplementary effect they must be given together and not on alternate days.

In comparisons on human subjects of the proteins of whole egg, yeast, cotton seed meal, corn (maize) germ flour, beefsteak and haddock, with mixtures of the essential amino acids, compounded in the proportions in which they occur in the proteins and supplying as much nitrogen as the protein, Murlin *et al.* (105) found that none of the mixtures possessed a biological value as high as that of the protein by from 10 to 40 per cent. The unnatural isomers, in so far as they escape deamination, belong to the class of dispensable compounds, while in so far as they are deaminated and can be recognised as contributing extra nitrogen to the urea and ammonia fractions of the urine, are in the same class as nonessential amino acids. The use of *dl*-synthetic amino acids, even those of the essential group, is nutritionally uneconomical for man.

*Effect of mustard gas.*—Exposure of casein to mustard gas rendered it inadequate to support growth of chicks and rats when fed as the principal protein source (106). Since the casein treated

with mustard gas was not toxic, the nutritional inadequacy was taken to mean either that the animals were unable to split the diethyl sulfide radicals (-$C_2H_4SC_2H_4$-) from certain essential amino acids or that certain amino acids became unavailable. Biological assay indicated that histidine, lysine, methionine, and threonine became unavailable for growth when the reaction was carried out at pH 9.3. At pH 7.3 the histidine, lysine, and methionine were still unavailable in hydrolysates of treated casein, but threonine now became available for growth. Rats were unable to utilize the cysteine or valine which had the radical, -$C_2H_4SC_2H_4$-, attached to the sulphur and nitrogen of these amino acids respectively. It would appear that dichlorethyl sulphide combines with the affected amino acids, excepting threonine, and that the nutritional inadequacy is due to inability on the part of the rat (or bacteria) to split the H-residues ($-C_2H_4SC_2H_4$) (107).

*Distribution of amino acids.*—Barton-Wright & Moran (108) estimated the essential amino acids in each of four fractions of specially milled mixed grist. The highest concentration of each amino acid was found in the germ and the lowest in the inner endosperm. The concentration of every amino acid was greater in the outer than in the inner endosperm. With arginine, lysine, and tryptophane the concentration in the bran was greater than in the outer endosperm, but the concentrations of the remaining amino acids were lower in the bran than in the outer endosperm, suggesting a diluting effect of the outer fibrous pericarp layer.

To gain information concerning the histological localization of amino acids and protein in the wheat grain and in the epicotyl and roots of the germinated grain, a survey of the available microchemical tests was made by Glick & Fischer (109). Tests for arginine involving a modification of the Sakaguchi test have been used (110, 111) for the purpose of locating this amino acid.

Although there does not appear to be any relationship between the concentration of an individual free amino acid and the total concentration of that amino acid in the plasma, the pattern of free amino acids in the plasma resembles that of the plasma proteins and of muscle protein (112).

*Acetyl derivatives.*—In 1910 Knoop (113) suggested N-acetyl derivatives as intermediates in the biological synthesis of amino acids. This suggestion gained support from the work of du Vigneaud & Irish (114) and their co-workers (115). Bernard (116) has

shown that acetic acid is a source of acetylation of phenylaminobutyric acid in intact animals and Bloch & Rittenberg (117, 118) have proved that pyruvic acid is also a source of acetyl. More recently Bloch & Borek (119) have made observations *in vitro* on rat slices using deuteroacetic acid and have found that acetylation of leucine and phenylalanine can take place. This strongly supports the suggestion that acetylamino acids are products in the normal intermediary metabolism of amino acids.

*Activation of deamination.*—In an investigation of the influence of different amino acids on the oxidative deamination of *d*-alanine by a purified preparation of *d*-amino acid oxidase isolated from the pig's kidney, it was found that when the concentration of enzyme was considerably reduced the breakdown of *d*-alanine was activated in the presence of *l*-leucine; other *l*- and *d*-amino acids, in particular, *l*- and *d*-histidine were effective. Peptides and proteins were also activators, their effect being more pronounced the greater the histidine content of the protein (120). When the activation of oxidative deamination of *d*-amino acids (other than *d*-alanine) was effected by *l*-histidine, it was found that the amino acids could be divided into two groups. The first group comprises those whose *l*-forms are only broken down to a small extent by tissue slices, and where the breakdown of the *d*-forms of these amino acids is strongly activated by *l*- and *d*-amino acids. To this group belong the monoamino monocarboxylic acids. To the second group belong the amino acids whose *l*-forms are readily decomposed by specific tissue enzymes and whose *d*-forms are only slightly activated in the presence of other *l*- and *d*-amino acids.

*Thio-amino acids.*—Cohn *et al.* (121) have found that the rate of methyl transfer from methionine is not proportional to the level of methionine in the diet. The demonstration of the growth promoting power of certain methylated amino acids (N-methylmethionine, N,N′-dimethylhomocystine (122), and N,N′-dimethylcystine (123)) was held to be tantamount to demonstrating that their keto acid analogues were also capable of supporting growth. It was thus inferred that the $\alpha$-keto acids derivable from homocystine, methionine, and cystine were utilizable for growth purposes. Since then the keto acid has been found to be effective in supporting the growth of rats on a diet deficient in methionine (124). In the conversion of a *d*-amino acid to its acetyl-*l* derivative,

the keto acid has been considered a likely intermediate followed by asymmetric synthesis of the *l*-acetyl compound. Wood & du Vigneaud (125) have isolated N-acetyl-S-benzyl-*l*-homocysteine from the tissue of a rat fed N-methyl-S-benzyl-*dl*-homocysteine and N-acetyl-S-benzyl-*l*-cysteine has been isolated from the urine of a rat fed N-methyl-S-benzyl-*l*-cysteine.

Shen & Lewis (126) have shown that oxidative deamination of the sulphur-containing amino acids or their derivatives may occur, even though further oxidation (i.e., of the sulphur to sulphate) is blocked by the presence of a non-labile substituent group (in their experiments, ethyl or benzyl) attached to the sulphur. By using radioactive sulphur ($S^{35}$) they have now been able to show (127) that sulphide sulphur can be used by the intact rat for the synthesis of cystine. Actually the amount of synthesis employing sulphide sulphur is small even when an active deposition of protein, as indicated by an increase in bodyweight, is proceeding.

The observations of Steensholt (128) suggest that the muscle and liver of the rat contain an enzyme system capable of catalyzing the transfer of methyl groups from methionine to ethanolamine, the ethanolamine being thereby converted into choline while methionine is demethylated to homocysteine.

Glynn, Himsworth & Neuberger (129) have shown that rats on a diet devoid of both cystine and methionine develop a massive hepatic necrosis and excrete a substance resembling homogentisic acid in the urine. If cystine is added necrosis does not develop, nor is this substance excreted. It has been found that deficiency of sulphur-containing amino acids decreases the ability of the body to metabolise aromatic amino acids to such an extent that even with small intakes of tyrosine and phenylalanine considerable amounts of these substances are excreted in the form of homogentisic acid (130). Cystine partially protects against this alcaptonuria. Small amounts of methionine without any cystine apparently do not protect effectively against hepatic necrosis. It is suggested that the important dietary factor which is responsible or partly responsible for the development of necrosis is the deficiency of cystine and that the protective effect of methionine is due to its being a precursor of cystine in the body.

Neuberger & Leaf (131) have found that the glutathione of the liver is very sensitive to dietary changes. It is concluded from the time relationships between the onset of necrosis and the reduction

of the liver glutathione content that the two phenomena are not necessarily related as cause and effect though reduction in the glutathione may be a contributory factor in the development of necrosis.

Wanscher (132) has found that on a casein-free diet rats develop a severe degeneration and necrosis of the liver with haemorrhage and intestinal inflammation. The necroses were chiefly central. In addition there were found degenerations in the convoluted tubules of the kidneys and occasionally the animals developed severe toxic and chronic convulsions. Furthermore, 5 to 10 per cent casein added to the diet gave no protection against the changes in the liver. Supplementation with 0.2 per cent cysteine did not protect whereas 0.2 per cent cystine prevented the liver changes but gave deficient growth. An addition of 0.2 per cent methionine gave normal growth and in these cases the liver was found to present no pathological changes. When 0.2 per cent cystine was given along with alcohol the animals developed a pronounced fatty liver.

Sydenstricker *et al.* (133) have found that rats on a methionine-deficient diet frequently develop corneal vascularisation. No corneal change was noted by one of these authors in the undernourished population of Holland in 1945.

Wingo & Lewis (134) have found that isocysteine is physiologically inert and is excreted in large part unchanged in the urine of rabbits to which it is administered. This compound is of interest not only because of comparison with the isomeric sulphur-containing compound cysteine, but also because of its $\beta$-amino group.

Observations by Bennett (135, 136) support the view that bacterial synthesis of unknown factors influencing homocystine utilization can take place in certain strains of rats and under certain experimental conditions, and the biosynthesis of methionine under these conditions has been demonstrated (137). Almquist (138) has recently reviewed the interrelations between choline, betaine, and methionine.

*Lysine, histidine and arginine.*—Turkey growers are frequently disturbed by the occurrence of an unusual extent of white colouring in the primary and secondary wing feathers of bronze poults kept in complete confinement, particularly when the diets contain a high percentage of certain vegetable protein concentrates. The syndrome has been prevented by adding lysine to the

diet, or by substituting protein concentrates high in lysine. The diet must contain approximately 1.1 to 1.2 per cent of lysine for normal feather pigmentation and to permit optimum growth of poults (139).

Albanese & Frankston (140) have shown that histidine is necessary for the growth of young rats and for the maintenance of adult rats. When histidine was withdrawn from the diet of rats in which crystalline amino acids were the sole source of nitrogen, there occurred a loss of weight, decline in haemoglobin and plasma protein production, atrophy of the thymus, a delay in spermatogenesis, narrowing of the epiphyses, corneal epithelial metaplasia and corneal vascularisation. In general, the changes found were milder than those previously noted with rats on a phenylalanine- or leucine-deficient diet, which is consistent with the ability of the growing rat to synthesise some of its histidine requirement.

In addition, it has been found that 2-thiol histidine, which is closely related to ergothioneine, is not converted into histidine by the rat, nor can the glyoxaline ring of histidine be made by the rat (141); $\alpha$-N-acetyl histidine, but not the corresponding benzoyl derivative, is available for growth of the rat.

The results obtained by Albanese & Frankston (140) in finding that histidine is essential both for growth and maintenance in the rat have been confirmed (142, 143). Using a microbiological method for the estimation of $\beta$-alanine both in the whole rat and in muscle extracts, it was found (142) that nearly all the $\beta$-alanine of the rat is contained in a nonprotein fraction of muscle and gives a measure of the carnosine and anserine content of muscle extracts. It was found that the $\beta$-alanine of muscle extracts was only slightly reduced after prolonged histidine deficiency, indicating that the sum of carnosine and anserine was only slightly reduced. Separate estimation of carnosine suggested a reduction in the concentration of this peptide (which is derived from histidine), while the methylated derivative appeared to be unchanged.

The intestinal tract does not appear to be the place where arginine is synthesized by the rat (144). Isotope studies on the rat by Bloch have yielded no evidence that the amidine nitrogen of arginine can be utilized for any reaction except for creatine formation. The amidine group is ultimately derivable from $\alpha$-amino nitrogen (145). In the pigeon the amidine group cannot be synthesized, even in the presence of citrulline (146).

*Tryptophane.*—Keller (147) has found that growing rats of

either sex exhibit sterility when given a diet lacking in tryptophane even over a short period of time.

Spector & Mitchell (148) consider that the inherent physiological effects of nicotinic acid and tryptophane, which are not dependent upon an increase in the intake of food, are probably not related to a modification in the synthetic capacities of the body, or of the organisms of the intestinal tract, but possibly to some unknown function in the body in which nicotinic acid and tryptophane participate interchangeably, analogous to the interchangeability of choline and methionine. Woolley (149) has recently shown that the growth inhibiting effect of 3-acetylpyridine in mice can be counteracted by tryptophane and suggests that maize may contain a structural analogue of nicotinic acid which competes with that vitamin just as 3-acetylpyridine does. In an effort to elucidate the relationship between nicotinic acid and tryptophane Krehl *et al.* (150) have found that nicotinic acid improves the utilization of tryptophane and it has been suggested that tryptophane improves nicotinic acid synthesis and this in turn plays a dual role in providing adequate nicotinic acid for tissue growth and improving tryptophane utilization. It is as yet uncertain whether tryptophane acts to stimulate nicotinic acid synthesis in the rat or whether it is an actual precursor of this substance, although the recent observations by Rosen, Huff & Perlzweig (151) indicate that tryptophane may be the important precursor in the synthesis of nicotinic acid.

As the result of metabolism studies on the fate of acetyl-*dl*-tryptophane given by mouth to human subjects, it was concluded that with the exception of a 5 per cent urinary loss all of the acetyl-*dl*-tryptophane might be available to man (152). More recently, however, experiments by Luck *et al.* (153) have shown that when acetyl-*dl*-tryptophane is administered intravenously to human subjects some 70 to 83 per cent is excreted unchanged within the first six hours after injection. The substance excreted was racemic. There was no preferential retention of acetyl-*l*-tryptophane. It would appear, therefore, that the route of administration plays a part in the intermediary metabolism.

*Serine.*—Artom *et al.* (154) found that when *dl*-serine was given to one group of rats and *l*-serine to another the former only developed typical renal lesions of serine intoxication. *dl*-Alanine did not have this injurious effect (155). Renal necrosis has also been noted by them to occur in young rats within twenty-four hours

after receiving a supplement of 100 mg. *dl*-serine to their stock diet (156). Healing of the necrosed tissue proceeded even if the serine administration was continued. The injurious effects of serine on the kidney were more marked if the diet was low in protein and the B group of vitamins, some of the animals apparently dying of peripheral circulatory failure.

*Glutamic acid and glutamine.*—It has recently been shown that the sulphoxide from *dl*-methionine is a growth-inhibiting analogue of glutamic acid for *Lactobacillus arabinosus*, with an antibacterial index of 75 (total inhibition) (157). Experiments indicate that methionine sulphoxide prevents the amidation of glutamic acid to glutamine. The sulphoxide may therefore be a useful weapon in the study of glutamic acid metabolism.

*Other protein derivatives.*—Womack & Rose (158) believe that there is in proteins an unidentified substance which, like arginine, is not necessary for fairly rapid gains, but which is required for maximum increases in weight. Whether the substance is identical with strepogenin remains to be established. According to Woolley & Sprince (159) the latter may be a peptide.

Starting with the hypothesis that the superior growth-promoting power is due to the higher strepogenin content, Woolley (160) has made a correlation between the strepogenin content of a number of proteins and their growth-promoting activity for mice maintained on a diet devoid of strepogenin. Deficiency of the growth-promoting substance in the basal diet does not lead to complete failure of growth or to death. The fact that it can be obtained from highly purified crystalline proteins suggests that it is a constituent of, rather than a contaminant of these proteins. The further information that it is released slowly by tryptic digestion of the proteins, is unstable to acid or alkali, and stable towards chloramine-T suggests that it is a peptide and not an amino acid. The inactivity of peptic digests of the proteins also supports this deduction.

The plasma of young adults contains some 0.1 to 2.2 mg. ($\pm$0.9 mg.) per 100 ml. of combined "nonprotein" $\alpha$-amino-nitrogen (161). Alanine and glycine contribute to the bound amino acids in about the same proportion as to the free. Human erythocytes contain concentrations of free $\alpha$-amino acids similar to those of plasma, and slightly more of combined acids including glutathione.

Florkin (162) has described the stages of both purine catabolism and protein catabolism in the same animal and in different

zoological groups. Various workers have consistently found high values for amino-nitrogen in the blood plasma of insects varying from 80 to 300 mg. per 100 ml. and to some extent with the life stage of the insect. In *Dytiscus marginalis* some 33 mg. histidine and some 138 mg. tyrosine per 100 ml., accounting for only a little over 8 per cent of the total amino-nitrogen, have been found (163).

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# ANTIOXIDANTS[1]

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Aside from the accepted effects of lowered temperature and accumulation of end-products, the retardation of chemical reactions follows from two causes, the disabling of a positive catalyst or the breaking of chains in a chain reaction. The concept of negative catalysis appears no longer to be acceptable; the potential energy barrier to the initiation of a reaction is lowered but cannot be raised by a catalyst.

The elimination of a catalyst is achieved in a variety of ways, sometimes specific, sometimes general, and is often mysterious enough that physical chemists have turned to biology for a suitable term, the "poisoning" of a catalyst. Biochemistry presents a bewildering array of catalysts, organic compounds of simple and complex composition, metallic ions and salts, upon which the progress of cellular reactions depends, and whose action can be antagonized by appropriate and usually very specific agents. These have been called metabolite antagonists, some of them are analogs of essential metabolites; their scope and the manner of their action have been reviewed recently (1).

Insofar as these agencies inhibit oxidation under natural and experimental conditions they might be called antioxidants. Cyanide would thus be antioxygenic for the reactions catalyzed by cytochrome oxidase; fluoride, iodoacetate, arsenate, malonate and many other substances which block enzyme-controlled oxidations might also be called antioxidants.

[1] The literature on autoxidation and antixodants is unbelievably extensive and the bibliography is in no sense complete. It is hoped that the significant contributions are at least cited in the reviews and original papers listed.

* For clarifying discussions of the subject, the writer is greatly indebted to the Josiah Macy, Jr. Foundation, to Dr. Paul György, to Dr. H. S. Taylor, and to all those who took part in the conference on physiological antioxidants, held in New York, October 10 and 11, 1946.

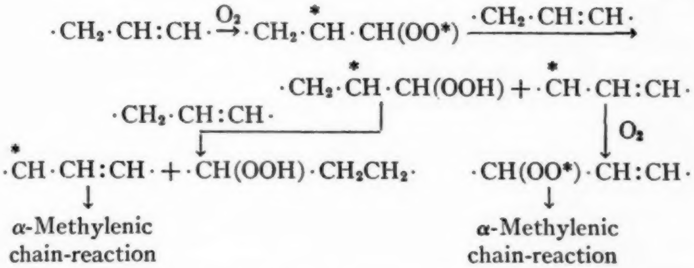
In its common usage, however, the term antioxidant implies inhibition of oxidation not by removal of a catalyst but by the breaking of reaction chains. Since these chains may be propagated by agents that are themselves catalysts, or pro-oxidants, the distinction is apparently not clear-cut, but it is assumed that pro-oxidants merely initiate more chains, which the inhibitor breaks. Autoxidative reactions have thus far rarely if ever found a place in the consideration of biological oxidation, doubtless because they get out of hand so easily. Rigorous control is a striking feature of the activities in normal living tissue.

*Chain reactions.*—An exposition of chain reaction mechanisms would be out of place in this review. The names of Christiansen (2), Moureu & Dufraisse (3), Semenoff (4), and Hinshelwood (5) are associated with the development of the theoretical and practical aspects. In the introduction to his book, Bailey (6) reminds us of "the loss, inconvenience and even calamity suffered continually through the occurrence of undesirable reactions, the oxidation of materials which are better unoxidized."

The examples from industry are many and of strategic importance, e.g., corrosion of metals, explosions, aging of rubber, knocking of fuels in internal combustion engines, rancidification of fats (7), and deterioration of chemicals and foods. Most of the recent theoretical advances have been made by fundamental researches in these applied fields. Biochemistry, whether of animals, plants, or their products, is primarily concerned with the autoxidation of lipids and lipid-soluble substances and with the deterioration of other constituents of tissues or of foods that may accompany fat spoilage, or occur independently of it, as a result of oxidation.

Innumerable studies have been made of the autoxidation of unsaturated fats. The formation of peroxides at the vulnerable double bonds has long been considered as the starting point but the results of recent research in fat and rubber chemistry have indicated a free radical mechanism. The $\alpha$-methylene group is the place of detachment of a hydrogen atom and the immediate point of origin of reaction chains.

The intermolecular scheme of Farmer (8) and Bolland & Gee (9) is in part as follows:




As thus formulated, addition of oxygen at a double bond begins the process, although at high temperatures (10) the extent of such addition is insignificant, and sufficient only to produce a free radical by detachment of an α-methylenic hydrogen atom. The resonating radical residues are responsible for the rearrangements in the chain, which produce conjugation (11) and polymerization. Stable α-methylene hydroperoxides have been isolated. Secondary reactions accompanying the increase and decay of peroxide account for the shorter chain scission products, including dibasic and aldehyde acids, which are the organoleptic evidence of rancidity.

*Autoxidation of fats.*—Natural fats and oils differ in their susceptibility to autoxidation depending less on the degree of their unsaturation than upon their content of pro-oxygenic and anti-oxygenic substances (12), their exposure to heat and light, the presence of traces of certain metals (13), especially copper and iron, and of hematin in lipid water systems (14). This susceptibility can be determined by several empirical methods in which light, heat, oxygen, or pro-oxidants (metal ions, hematin, carotene) are employed to accelerate the process, which can be measured by the rate of oxygen uptake in closed systems, the production of peroxides or of volatile oxidation products or the bleaching of natural pigments (such as carotene) or added dyes (methylene blue). The plotted curves are always *S*-shaped even though they cannot be sufficiently magnified to show this. The rapid progress of the reaction is preceded by a period of relative inaction, known as the induction period, during which the primary reaction of chain formation is proceeding at the same maximum rate as later but the chains are being broken as fast, or almost as fast, as they

are started. Some are broken by collision with the walls of the containing vessel, by far the most of them are broken by encounters with antioxidant molecules. With the exhaustion of these, increasing numbers of chains run their course. The chain breaking action of antioxidants must therefore be exerted on the activated chain starting radical, or even earlier, before this radical is formed by contact of an excited methylene group with molecular oxygen.

*Antioxidants.*—Although the biochemical implications of antioxidants first became obvious with the advent of vitamin E, the capacity of certain natural products and common chemical substances to delay the rancidification of fats had long been known and utilized. More recently other naturally occurring inhibitors have been found and tested (15, 16, 17), among them, polyphenolic substances, some of which are glucosidic in origin, and certain sulfur compounds in the presence of water, such as sulfhydryl compounds (18), thiourea (19), and thiodipropionic acid (20). The first qualification for such agents is apparently the possession of a labile hydrogen. The manner of action of the sulfur compounds has apparently not been studied but among the phenolic substances, those having hydroxyls in the ortho or para position (or an equivalent electronic configuration) are most effective (21). Another qualification is the possession of a redox potential within a relatively narrow range (22), the reason for which is not immediately apparent. When two of them, used together, have an amplified action (synergists), the one with the higher potential is within the effective range.

When the structure of the tocopherols became known their antioxygenic behavior was quite in accord with what should be expected of substituted quinols. As compared with the chroman nucleus the tocopherols are less effective because of nuclear alkylation (23) and the long hydrocarbon side chain; in the usual accelerated tests, the $\alpha$-compound (trimethyl) with the highest biological activity has the least antioxygenic activity.

When the tocopherols are oxidized in fulfilling their function as antioxidants, the reaction appears to be of the first order (24) and to take place in the accepted two steps (25), the first of which may be reversible and perhaps of biological significance; the quinone and hydroquinone lack biological activity. The vicarious oxidation of vitamin E is in part responsible for the original discovery of vitamin E as essential for reproduction and other physiological processes.

Another group of stabilizing agents, commonly called acid inhibitors, includes certain organic and inorganic acids which have little if any antioxygenic action on purified fatty acids and esters but which powerfully reinforce the action of the phenolic inhibitors present in vegetable oils or added as such to animal fats ordinarily devoid of them (26). Two of these, ascorbic (27) and phosphoric (28) acids, have received attention as to the manner of their action; it has been assumed that by a combination of adsorption and solution reactions, they donate electrons and protons to quinones to replace those lost by quinols in the breaking of reaction chains and thus insure a continuing supply of phenolic antioxidants. More recently (29) a mechanism based on exchange reactions has been proposed. Phosphoric acid has been utilized ingeniously as an exchange agent in olefin chemistry (30); its effectiveness depends on the presence of hydroxyl groups which by hydrogen bonding allow shifting (and therefore de-excitation) of ethylenic bonds. In fats containing quinols, the formation of similar hydrogen bonds between a phosphoric acid hydroxyl and resonating quinone structures, arising from antioxygenically "spent" quinols, transforms the phosphoric acid into a lipid soluble substance whose remaining hydroxyls are thereby made available throughout the lipid phase for hydrogen bonding with ethylenic structures. However valid this scheme will prove to be, it offers the first acceptable basis for explaining why certain acidic substances, among them di- and tribasic organic hydroxy acids, ascorbic acid and pyruvic acid, have a stabilizing action on fats in the presence of quinols. The degree of acidity required for the ionization of the antioxygenically active acids makes it unlikely, at the moment, that they play a similar role in living tissue, but the biochemical study of free radicals and exchange reactions is in its infancy.

Not yet identified are the characteristics of certain other antioxygenic agents found in nature. Among these are substances released from seed cakes by organic acids (31). Thermolabile inhibitors occur in various cereal grains; oat flour has been used for the stabilization of many food products whose fats are susceptible to oxidative spoilage (32). The polymerization products of heated sugars, perhaps dienols, have been proposed for use in a great variety of combinations (33). Since they originate under the same conditions which destroy some inhibitors and predispose fats to early rancidification, it is not surprising that the stabilization of a composite food containing carbohydrate and protein as well as

fat, such as crackers, has presented baffling problems (34). The browning of frozen fruit, which is prevented by ascorbic acid (35) is an interesting example of oxidative deterioration that occurs independently of changes in fat. Epinephrine, a powerful inhibitor of fat autoxidation and itself easily oxidized, is stabilized by ascorbic acid (36).

Crude sources of the vitamin B complex also contain agents which delay the oxidation of unsaturated fat acids and the decolorization of carotene (37); among them are yeast, liver extract, molasses, and rice bran extract. [Cane molasses was early shown to contain vitamin E (38).] The active material in rice bran extract was relatively thermostable; although dialyzable it was somewhat soluble in fat, and synergic with tocopherols. Replacement of yeast by a mixture of pure preparations of the B vitamins allowed the experimental diets to become rancid (39), but this mixture did not include *p*-aminobenzoic acid or biotin; the former has fat-stabilizing action (37) and since the latter is destroyed by rancidifying fats (40) it can, presumptively, also act as an antioxidant upon enolization.

Doubtless there are other, perhaps vitamin like, constituents of yeast and liver that disappear early in the process of rancidification. A dermatitis-preventing factor destroyed by rancid fat (41), the secondary anemia seen in animals on diets high in linoleic acid (42) and the dermatitis occasionally alleviated by wheat germ oil (43) are further suggestive examples of the importance of the stabilizers found among the noncaloric components of fats (44). Since there is no indication that their action is physiologically specific, the implications in the term co-vitamin may be questioned.

Antioxygenic substances are thus of varied nature; for the most part, they are themselves oxidizable and may, under suitable conditions, actually facilitate rather than delay oxidation (45). These facts readily account for the many conflicting and unexplained observations that have been made on the stabilization of different substrates under conditions differing as to concentration of inhibitor, temperature, aeration, illumination, amount of moisture, and the unsuspected presence of natural pro-oxidants and synergists.

*Tocopherols as chemical antioxidants in vivo.*—Of the naturally occurring phenolic antioxidants the tocopherols are at once the

most abundant and the most important, biochemically. Even before they were known as such, concentrates of tocopherols were called inhibitols (46). Added to the vegetable oils from which they come, they are progressively less effective and exhibit an optimum concentration (24) characteristic of inhibitors, and thus offer another of those specious teleological arguments for the eternal fitness of nature.

Deprivation of vitamin E is followed by a bewildering array of physiological abnormalities in different species (47, 48). Female rats and mice demonstrate resorption gestations; male rats, but not male mice, suffer testicular degeneration; muscular dystrophy appears in nursling rats and mice but if they survive it, they appear to grow normally and there is a long delay before muscle dystrophy reappears. On the other hand, young rabbits and guinea pigs, dogs and hamsters succumb to muscle degeneration between weaning and adolescence; lambs develop "stiff lamb" disease; ducklings become dystrophic, but chicks develop cerebellar encephalomalacia and a subcutaneous exudative diathesis along with muscle changes; in turkeys there is selective necrosis of the smooth muscle in the gizzard; the myopathy in mammals is accompanied by a high urinary creatine excretion, a decreased muscle creatine, an increase in muscle chloride and calcium, certain changes in lipids and an increased consumption of oxygen by muscle strips *in vitro*.

If these diverse manifestations of vitamin E deficiency could all be causally related to the presence of nonphysiological fatty acid peroxides, the role and manner of action of vitamin E could probably be explained on a chemical basis more simple than that of any other of the vitamins.

It has been pointed out (49) that in vegetable tissue the highly unsaturated lipids and lipid-soluble pigments are well protected from autoxidation by the cell walls and by the stabilizers associated with them. All of the natural antioxidants originate in plants; with the exception of cephalin, they cannot be synthesized in animal tissue. In the animal organism there are no barriers to the diffusion of oxygen but, nevertheless, unsaturated fats do not normally undergo peroxidation. Under usual dietary conditions perhaps animals obtain adequate amounts of stabilizers in their food. Only minute amounts are needed since the process is a chain reaction, but for the same reason, once the process has begun, it is

the more difficult to stop. In any case, to produce the disorders of vitamin E deficiency, special nutritional regimes are required, characteristic of which, aside from paucity in vitamin E, is a high intake of unsaturated fats. Long before tocopherol was known, Agduhr (50) had described cod liver oil damage in heart tissue. After it was demonstrated that vitamin E disappeared during the induction period of rancidifying fats (51), the "anti-vitamin" effect of highly unsaturated fats could be explained on the basis of the autoxidative destruction of vitamin E. It still remains to be proved, however, whether the absence of vitamin E, *per se*, is the direct cause of the damage or whether fat peroxides are the cause and can merely accumulate in the absence of a suitable stabilizer. Is vitamin E solely a chemical antioxidant or does it, above and beyond that, also have a physiological role?

Kudryashov (52) took an apparently middle ground by saying that toxic oxidation products of fatty acids or possibly of sterols play the major role in the production of sterility in rats, quite independently of vitamin E; its presence greatly reduces their toxic action and may prevent their appearance but there is no chemical reaction between them and vitamin E. The bioassay method is very circumstantial and he was dealing with borderline deficiencies, but if, in the strict sense, the function of an antioxidant is to prevent the formation of peroxides rather than to destroy those already formed, his interpretation may be quite valid.

From observations on chicks (53) Dam concluded that the most highly unsaturated fats were most damaging and that slightly rancid fats produced exudative diathesis whereas thoroughly rancid fats did not (54); the latter always contain less peroxides than the former. He was also able to demonstrate peroxides in the adipose tissue of some chicks fed cod liver oil (55) but the amounts were extremely variable and showed no correlation with the extent of exudate. $\alpha$-Tocopherol, but not $\gamma$-, prevented peroxide formation. Results with rats were even less conclusive.

That vitamin E has antioxygenic action, in the purely chemical sense, *in vivo* as well as *in vitro*, is beyond question. The sparing action of vitamin E on vitamin A in growing rats, first demonstrated by Moore (56), and verified by many others as regards both vitamin A and carotene (57), indicated a protecting effect in food

and in the alimentary tract. This was further confirmed by the physiologically important lipophilic character of tocopherol (58) and by the advantage gained from feeding it separately to animals after a few hours of fasting (59). Proof that this protection extends to the tissues is found in the low stability of the body fats of rats on an E-deficient diet, in the selective deposition of tocopherol in these fats, thereby greatly prolonging their induction period *in vitro* (60), and in the sparing action of tocopherol on the essential unsaturated fatty acids (61).

Since liver and muscle are preferential sites of storage as compared with depot fats (62), the latter are not likely to be enriched until the former have been well supplied. The supply of oxygen to the fat depots is also much less than that to active functional tissue, as if the allocation of antioxidant to tissues were made on the basis of probability of autoxidation. Such arguments from design are superficial and sometimes dangerous. Nervous tissue contains a high proportion of unsaturated fatty acids but except in the chick, the nervous system is not known to be primarily affected by lack of vitamin E. Some information is at hand on altered lipid distribution in nervous tissue in vitamin E deficiency but none on the normal content of vitamin E and its variations.

The argument that peroxides may never appear in tissues unless the organism is flooded with unsaturated fats, or is deprived of tocopherol, may be weighed in relation to the acid-fast brownish pigment which appears in various tissues of E-deficient animals. It was first observed by Martin & Moore (63) in the uterus and kidneys of rats kept on a vitamin-E free diet and is now known to appear also in skeletal and cardiac muscle, in lymph nodes, spleen, and adipose tissue, especially if the diet contains large amounts of cod liver oil. Mason *et al.* (64) have made extensive histological, physiological, and chemical studies of this pigment, concluding that in fatty tissue, at least, it consists of polymerized products of long chain unsaturated fatty acids produced as the result of peroxidation. Since polymerization is the result of preventable but not reversible free radical formation, the prophylactic value of vitamin E is understandable, as well as its lack of effect on previously deposited pigment. In this connection, myocardial lesions in vitamin E deficient animals have also been demonstrated by functional tests (65).

As judged by its fluorescence, this pigment appears not to be

identical with the ceroid pigment of nutritional cirrhosis (66) produced by a diet low in methionine and choline, and preventable by added choline. Since cirrhosis without ceroid can be produced in animals on fat-free diets (67) and since the diets on which ceroid appears are characterized by a high content of cod liver oil (68, 69) or the absence of crude sources of the vitamin B complex (68), it is reasonable to conclude that any accumulation of unsaturated fats, from whatever cause, may involve the risk of peroxidation unless it is accompanied by adequate amounts of stabilizing agents.

The interesting antistiffness factor for guinea pigs found by Wulzen, vanWagtendonk *et al.* (70) in raw cream and cane juice is fat-soluble and very susceptible to oxidation. The presence of iron and copper salts in the experimental diet can not be unrelated to the deficiency, and the inclusion of cod liver oil hastens it. Although the manifestations of deprivation and the distribution and chemical characteristics of the factor are not the same as those of vitamin E, the complicity of autoxidative destruction is highly probable.

*Tocopherols as physiological antioxidants.*—Several considerations do not favor the simple explanation that vitamin E is no more than a chemical antioxidant in living tissue. In its absence dystrophy can be produced in rabbits on practically fat-free diets (71). However, such animals are not fat-free; perhaps minimal amounts of vitamin E are necessary to prevent autoxidation, even of endogenous fat.

The high degree of specificity of $\alpha$-tocopherol is a further obstacle to this simple explanation. Not only is it less effective than the other tocopherols as an antioxidant but tampering with its structure lessens its biological efficacy (72); antioxidants within the same range of redox potential are biologically inactive (73). Absorption from the alimentary tract is certainly a consideration (58), and information on this subject should soon be available if it is possible to employ the modified bipyridyl method which takes advantage of the slower rate of color development with $\gamma$- as compared with $\alpha$-tocopherol (74). In addition to differences in solubility, optimum temperature may also be a factor. If the greater stabilizing action of $\alpha$-tocopherol at low temperature (30°C.), as compared with that observed in thermally accelerated tests, is confirmed, and

if the basis for differentiating between antioxygenic potency and activity is valid (75), the discrepancy between the chemical and physiological findings may be removed.

The most difficult feature of the attempt to use the antioxygenic action of tocopherol as the key to unlock the secret of all of its functional roles is the great diversity of these roles. How can any key, even a master key, fit so many different kinds of locks?

The departure from normal physiological behavior induced by a lack of vitamin E has been studied especially in the skeletal muscles of certain species. Pappenheimer's comprehensive review (48) of this structural and functional impairment summarizes all the pertinent literature into 1942. The appearance of paralysis in nutritional muscular dystrophy is coincident with severe structural damage, with altered chemical composition, and with a decline in functional efficiency that may not be closely correlated with the other changes and probably precedes them in point of time. Tocopherol is effective preventively, and under watchful treatment also curatively.

These dystrophic muscles have a higher oxygen consumption than normal muscles. The initial observation by Victor in 1934 has been confirmed repeatedly but with a disconcerting lack of uniformity, perhaps associated with varying degrees of degeneration from one animal to another or from one muscle to another in the same animal. Intravenous administration of tocopherol phosphate reduced the uptake of oxygen to normal within a few hours as shown by biopsy experiments on rabbits (76). Kaunitz & Pappenheimer (77) found that a single dose of 1 mg., given to a nursling vitamin E-deficient rat, produced a lowered oxygen consumption not only of the isolated muscle, as compared with controls, but also of the entire animal throughout the period of adolescence. A disturbing feature of their observations was the higher oxygen consumption of isolated muscles from nursling rats of mothers on stock diets, higher than that of E-deficient rats. In his search for the underlying mechanism Houchin (78) succeeded in lowering the *in vitro* oxygen uptake of dystrophic muscle, and the succinic dehydrogenase activity of homogenates prepared therefrom, by the addition of $\alpha$-tocopherol phosphate (not tocopherol) to the nutrient medium. The latter observation has not been confirmed (79, 80); succinic dehydrogenase preparations from normal and dystrophic

muscles are alike in activity and this activity is equally depressed by tocopherol phosphate and tocopherol succinate but not by the insoluble calcium succinate ester of tocopherol.

A recent interpretation (81) of the role of tocopherol phosphate, based on present knowledge, is that the increased concentration of calcium in dystrophic muscle may be responsible for an enhanced adenosine triphosphatase activity, and this, in turn, for the increased oxygen uptake; tocopherol phosphate may precipitate calcium ion. At the moment, solubility rather than phosphorylation appears to be the essential property. Tocopherol phosphate is hydrolyzed in the organism (82, 83) but we have no notion as to the form in which tocopherol is active biologically; a complex with phosphoric acid other than an ester may exist (29). There is no information as to which if any of the sources of energy is being burned preferentially in dystrophic muscle or as to the significance of the loss of creatine and creatine phosphate and of the other alterations in composition. In particular, information is needed on the presence of fat peroxides; they profoundly influence chemical reactions *in vitro* (84).

Whether vitamin E is a member of the enzyme systems governing metabolism in muscle tissue, or whether it merely stands by, as a guard, to prevent the rise of peroxides, cannot be known until some of these questions are answered.

*Carcinogens.*—Since chain reactions are not subject to control, once they get a start, it is perhaps not surprising that students of cancer have been interested in antioxidants, but the observations thus far reported cannot be clearly interpreted. Carcinogenic hydrocarbons inhibit catalyzed phospholipid oxidation and also the autoxidation of unsaturated fats, being themselves destroyed in some cases via quinone formation (85). The more rapidly they disappear in such coupled oxidation the less effective they are as carcinogens. By the same token their action can be increased and prolonged if they are protected by other antioxidants. Of special interest are the experiences with butter yellow (N,N-dimethylaminoazobenzene) whose carcinogenicity was decreased by feeding it in a diet containing unsaturated fats, especially linolein, or in a diet low in pyridoxine (86) and was increased by diets containing rice, or Crisco, or butter fat (42), or by the addition of biotin to an otherwise protective diet (87). The butter yellow was apparently protected from oxidation by the antioxygenic effect of biotin, the

tocopherols in Crisco and butter fat, and the unidentified stabilizers in rice. More recent observations (88) cannot be so interpreted and in any case, even if other malignancies behaved like this experimental type, as they surely do not, the difficulty, not to say danger, of impoverishing the host of some of these vitamins hardly favors this approach to treatment. Numerous other but more equivocal examples could be given of the possible participation of pro- and antioxygenic agencies and autoxidizable substances in normal and abnormal animal processes.

Plant physiologists, in whose realm antioxidants originate, have found some of them (catechol, ascorbic acid) to be substrates in fairly well-defined oxidation reduction systems under the control of specific enzymes. The rapid chemical changes which take place when some plant tissues are exposed to oxygen can be prevented by ascorbic acid; in such case, ascorbic acid is presumably acting as a chemical antioxidant. Lipoxidase accelerates the peroxidation of fats but its action is delayed by phenolic antioxidants (or related compounds), presumably acting as such in the chemical sense. When biochemists have gained a clearer conception of the sequence of events in animal tissues, these events will probably also be explicable either as chain reactions or as processes catalyzed by enzymes.

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# CHOLINE

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The present review is principally concerned with articles which have been published recently, but references are sometimes made to earlier experiments and to their meaning in the light of recent discoveries. For further discussions, the reader is referred to an extensive review of choline (1) and to a detailed account of the reactions involved in biological transmethylation (2).

Recent studies with isotopically tagged molecules have clarified the nature of certain biochemical relationships between choline, methionine, and compounds which are concerned in reactions involving these two substances. It appears that methionine can yield a "labile" methyl group which can unite with aminoethanol in the metabolism of certain species to form choline. Aminoethanol can apparently be formed from glycine. Thus methionine can assume the important function of a dietary substitute for choline in these species. Choline, however, cannot replace methionine in the diet unless the amino acid homocystine (or homocysteine), which is not known to occur in foods, is added to the diet. When homocystine and choline are added to the diet it appears that a "labile" methyl group may be released from choline and unite with homocystine to form methionine. It thus seems that either choline or methionine can function as "methyl donors," and the question of "methyl acceptors" becomes of interest. The role of methylaminoethanol and dimethylaminoethanol as "methyl acceptors" and hence as biological precursors of choline has been studied with various species.

*The labile methyl group and quaternary nitrogen.*—Rats were fed a "labile-methyl-free" purified diet supplemented with homocystine and dimethylaminoethanol. The slow growth rate indicated that dimethylaminoethanol, in contrast to choline, did not supply methyl groups for the synthesis of methionine from homocystine at a rate sufficient to permit rapid growth on a diet devoid of methionine (2, 3, 4). However, when deuteriodimethylaminoethanol was fed, the choline subsequently isolated from the tissues was found to contain deuteriomethyl (4). These results strongly

indicate that dimethylaminoethanol functions as an acceptor of methyl but not as a donor of methyl to homocysteine. In terms of this line of thought, the quaternary nitrogen compound choline in yielding a "labile" methyl group is transformed into the tertiary nitrogen compound dimethylaminoethanol, the methyl groups of which are apparently not "labile." The concept of associating the lability of methyl with its attachment to quaternary nitrogen finds additional support in the observation that betaine, but not dimethylglycine or sarcosine, can promote the growth of rats on a "methyl-free" purified diet supplemented with homocystine (5). This may be taken to indicate that betaine, in yielding a "labile" methyl group, is possibly transformed into dimethyl glycine, a tertiary nitrogen compound. This compound does not readily yield "labile" methyl (6) and in this respect is resembled by sarcosine (150). It is evident that not all methylated quaternary nitrogen compounds can yield effective quantities of labile methyl, for homocholine, as an example, is inactive in promoting the growth of rats on a diet lacking methionine but supplemented with homocystine (5). It is also evident that the metabolic degradation of betaine cannot end with the formation of dimethyl glycine, for $N^{15}$ was found in tissue glycine following the feeding of betaine tagged with $N^{15}$ (6, 7). Quantitative studies (8) indicated that betaine under certain conditions was only about one-third as effective as choline in the prevention of the hemorrhagic kidney syndrome in rats. Among the possible explanations for this observation it was suggested (8) that perhaps only one of the three methyl groups of betaine was labile. This suggestion would fit in with the subsequent observations (5, 6) regarding the lability of methyl in betaine and its nonlability in dimethyl glycine.

Figure 1 illustrates the steps involved in some biological reactions concerning choline.

*Step 1.* Evidence has been provided (9, 10) for the oxidation of choline to betaine aldehyde by rat liver slices, and it was also shown that arsenocholine is similarly oxidized to arsenobetaine aldehyde (11). The enzyme system involved in the oxidation was found to consist partly of a dehydrogenase and partly of the cytochrome-indophenol oxidase system (11). The inability of betaine aldehyde to replace choline in purified diets for the chick (12) may indicate that step 1 is not readily reversed in this species.

*Step 2.* Studies of the choline-oxidation-enzyme system in rat liver preparations showed that when betaine aldehyde was added, oxygen uptake was increased with the possible production of betaine (9). It was shown (10) that betaine aldehyde was oxidized, probably to betaine, by rat liver brei at pH 7.8.

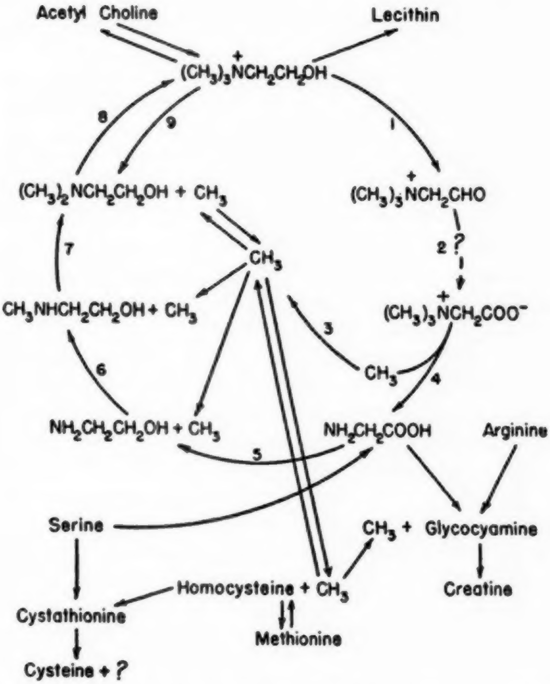


Fig. 1.—Diagrammatic illustration of the biological interrelationships between choline and its derivatives or precursors.

*Step 3.* Betaine has been found to yield labile methyl groups in the rat (3, 6) in experiments in which deuterium was used as a tracer element. Experiments on the growth of chicks (13) showed that betaine will promote growth when added to a labile-methyl-deficient diet containing homocystine, which suggests that betaine may yield labile methyl for the methylation of homocystine in this species as well as in rats.

*Step 4.* Glycine rich in $N^{15}$ was isolated from the tissues of rats to which betaine labeled with $N^{15}$ had been fed (6, 7). This provided definite evidence for the transfer of the nitrogen atom of betaine to glycine in the rat, although the pathway of this conversion is unknown. In the chick betaine does not appear to be an effective dietary source of glycine as judged by the inability of betaine to promote growth in chicks on a diet deficient in glycine (14).

*Step 5.* When glycine labeled with $N^{15}$ was fed to rats, the aminoethanol isolated from the body phosphatides had an isotope concentration 49 per cent as high as that of the glycine isolated from the body proteins (7). This indicated conversion of glycine to aminoethanol in the body.

*Step 6.* Studies with rats which received aminoethanol tagged with $N^{15}$ indicated that under the conditions of the experiments 41 per cent of the choline in the phosphatides was formed by the methylation of aminoethanol (7) presumably by the uptake of methyl groups derived from methionine (15). The chick does not appear to carry out this step, for this species is unable to utilize aminoethanol as a substitute for choline in a purified diet even when ample methionine is present (16). Mutant No. 34486 of *Neurospora crassa* similarly appears unable to utilize aminoethanol in the presence of methionine (17, 18). A Type III pneumococcus was found to utilize aminoethanol as a substitute for choline for growth on a purified culture medium (19).

*Step 7.* Rats were fed methylaminoethanol labeled with deuterium in the methyl group. Choline isolated from the tissues of these animals was found to contain a significant amount of deuterium in the methyl groups (4). As a replacement for choline, methylaminoethanol was found to be utilized by the chick for the prevention of perosis on a purified diet which contained methionine (18, 20), and by mutant No. 34486 of *Neurospora crassa* for growth on a purified culture medium (18, 20, 21).

*Step 8.* The evidence for this step was obtained with rats (4) by labeling dimethylaminoethanol with deuterium in the methyl groups, feeding the compound to rats, and subsequently demonstrating the presence of deuterium in the methyl groups of choline obtained from the tissues of the rats. Chicks (18, 149) and *Neurospora* mutant No. 34486 (21, 22) were found to utilize dimethylaminoethanol quite effectively as a substitute for choline.

*Step 9.* The evidence for the formation of dimethylaminoethanol from choline depends upon the observation that choline, but not dimethylaminoethanol, is an effective methyl donor as indicated by ability to promote growth in rats on a homocystine-supplemented diet (4). From this it may be deduced that choline forms dimethylaminoethanol in yielding a labile methyl group.

The formation of acetyl choline from choline, and the reverse reaction, are respectively catalyzed by two enzymes, choline acetylase (23) and cholinesterase. The formation of lecithin from choline was shown by observing the entrance of dietary choline, tagged with $N^{15}$, into the tissue phospholipids (24). The demonstration of the transfer of methyl from methionine to glycocyamine with the formation of creatine, and the formation of glycocyamine from arginine and glycine have been adequately reviewed elsewhere (2). Studies with rat liver slices incubated in a medium containing glycocyamine (25) indicated that the formation of creatine was accelerated when homocystine and choline were added together, but that there was no acceleration when either homocystine or choline were added alone. The evidence for passage of the sulfur atom of methionine, via homocysteine and cystathionine, to cysteine, and the transfer of the nitrogen atom and presumably the carbon chain of serine, via cystathionine, to cysteine, were reviewed (2, 26). Evidence for the occurrence of the reverse process in a lower organism, namely the formation of methionine from cysteine via cystathionine and homocysteine in *Neurospora crassa*, was recently presented (27).

*Nutritional relationships between choline and methionine.*—These may be summarized as follows: (*a*) In certain species, including rats, mice, dogs (28, 29), and probably man (30) methionine can replace choline in the diet and thus prevent the signs of choline deficiency. This replacement appears to be due to the biological formation of choline by the union of labile methyl groups with aminoethanol which may be formed from the dietarily dispensable amino acid glycine. Betaine may substitute for methionine in furnishing the labile methyl groups for this reaction (6). The synthetic compound, dimethylthetin, may also function in this manner in the rat (2); (*b*) Chickens on a purified diet are unable to utilize methionine or betaine as a dietary replacement for choline unless methylaminoethanol or dimethylaminoethanol are present in the diet, presumably because these animals appear to be unable to

"add" methyl to aminoethanol (16); (*c*) Choline or betaine have not been shown to prevent methionine deficiency unless homocystine is also added to the diet.

*The dual nutritional role of choline.*—Various considerations enable the functions of choline in nutrition to be divided into two broad categories. In the first of the two classes may be placed the function which appears to depend on the utilization of the intact molecule of choline (31) and possibly on its ability to form lecithin (32). This function is independent of the ability of choline to yield labile methyl and may include the effects of choline in preventing fatty livers (31) and hemorrhagic kidneys in rats and mice. The antiperosis effect of choline in chicks and turkeys and the growth promoting effect of choline on mutants No. 34486 and No. 47904 of *Neurospora crassa* may also fall into this class. The evidence in favor of this hypothesis may be expressed as follows: (*a*) Arsenocholine has lipotropic, anti-kidney-hemorrhagic and antiperotic properties (16, 33). Arsenocholine does not effectively promote the growth of rats (34) or chicks (35) on a labile-methyl deficient diet supplemented with homocystine, which indicates that arsenocholine does not function as a source of labile methyl; indeed, it seems possible that arsenocholine forms trimethylarsine in the body (11, 31); (*b*) Triethylhydroxyethylammonium (triethylcholine) chloride similarly has lipotropic (36) and anti-kidney-hemorrhagic (37) properties, and it does not promote growth with homocystine (38). The triethylcholine analogue of lecithin has been found in the tissues of rats which were fed triethylcholine (39); (*c*) Methionine and betaine have lipotropic and antikidney-hemorrhagic properties for the rat and the mouse which may be explained by the ability of the molecule of each of these substances to yield a labile methyl group for the methylation of aminoethanol to form choline in these animals. Arsenobetaine, which appears not to be an effective source of labile methyl (5), and which hence cannot function as a precursor of choline, is inactive in the prevention of fatty livers (31) or hemorrhagic kidneys (40).

The second function of choline depends upon the ability of its molecule to furnish a labile methyl group. This group may be transferred to homocysteine to form methionine and thence to glycocyamine to form creatine (4, 25). Betaine may assume this function in rats (6), and probably in chicks (13) although, like

methionine, it is ineffective in replacing choline for the prevention of perosis in chicks on a purified diet (12). The intact molecule of choline, rather than a supply of labile methyl groups, may be assumed to be needed for the latter purpose.

*The dual nutritional role of methionine.*—A predictable conclusion is that methionine has a dual nutritional role for the rat: first, as a building stone for tissue, either *per se* or as a precursor of cystine; second, as a source of labile methyl for the formation of choline and for the methylation of other compounds. This conclusion is supported by observations which differentiated hepatic hemorrhage and necrosis, prevented by cystine and methionine, from hepatic cirrhosis which was prevented by choline and by methionine as a choline presursor (41, 42). Along similar lines are other observations (43) that rats on diets of corn starch or lard with dried yeast as the sole source of protein developed "massive hepatic necrosis" which was prevented by casein, 8 per cent in the diet, or by 20.5 mg. of methionine daily but not by the small amount of 4 mg. of choline daily, and that this condition may be differentiated from "diffuse hepatic fibrosis" which was reported to result from long-continued heavy fatty infiltration of the liver (44) and which was produced by diets deficient in lipotropic factors. The authors expressed the opinion that the correct distinction was not made elsewhere (41) between diffuse fibrosis and "postnecrotic scarring." However, this opinion appeared to be founded on the belief that diffuse hepatic fibrosis cannot be produced by dietetic means in rats in less than 150 days. As an opposite standpoint is the general observation that the appearance of nutritional deficiency lesions may usually be hastened by modifications in the dietary regime.

An attempt was made to measure the amounts of methionine required respectively for growth and for the prevention of fatty livers in the rat. On a low-choline diet containing 0.1 per cent of cystine, it was stated that optimum growth was obtained with 0.6 per cent of methionine; however, the experimental data indicated that an increase in growth was obtained when the methionine content was further increased to 1.0 per cent. There was a steady decrease in the fat content per 100 gm. of liver tissue with increasing levels of methionine, and an approximately normal level of fat content was reached at a methionine level of 1.2 per cent in the diet. A slight decrease in the liver fat was observed when the

cystine level was increased and the methionine level was held at 0.5 per cent (45). The effect of cystine in these experiments may have been due to a "sparing action" upon methionine (46). It does not seem possible to differentiate the growth-promoting from the lipotropic effects of methionine in rats on a low-choline diet. On such a diet, a growth response to methionine is accompanied by a decrease in liver fat. It may be presumed that no sharply preferential affinity for methionine is possessed by the biochemical reactions concerned with protein synthesis as compared with the reactions occurring in the transformation of methionine to choline.

Observations on the effect of diet on liver fat emphasized the importance of nutritional adequacy, especially with respect to the essential amino acids, in making controlled observations on lipotropic effects (47).

It would appear important in nutritional studies involving protein deficiencies in rats to take cognizance of the fact that, unless adequate amounts of choline are supplied, a low-protein diet may bring about an acute deficiency of choline superimposed on a deficiency of essential amino acids. A level of 0.2 per cent of choline in the diet will probably supply a sufficient margin, if experiments with chicks can serve as a guide. On high-protein diets, the choline requirement of rats may usually be largely satisfied by the methionine present in the diet.

## Choline in Poultry Nutrition

On choline-deficient diets, perosis was prevented in chicks by methylaminoethanol or dimethylaminoethanol but not by aminoethanol (18, 20). It was suggested that chicks lack the ability to transform aminoethanol into methylaminoethanol, which would explain the ineffectiveness of methionine in replacing choline in purified diets for chicks, in contrast to the effect of methionine in replacing choline in the nutrition of dogs and rats.

Chicks were fed a diet which contained a soybean protein concentrate and which was deficient in cystine, methionine and choline (13). When methionine was added to the diet, there was an improvement in growth. A similar improvement was produced by homocystine plus betaine and, to a somewhat lesser extent, by cystine plus betaine. A more marked improvement was produced by any one of the following combinations: cystine, homocystine, and choline; cystine, methionine, and choline; cystine, methio-

nine, and arsenocholine; cystine, homocystine, betaine, and arsenocholine. The observations are consistent with the circumstance that the basal diet was deficient in both methionine and choline; with the dietary need of the chick for both of these substances, except that methionine may be replaced by homocystine plus a methylating agent such as choline or betaine; and with the previous finding (12) that arsenocholine can serve to replace choline for the chick on a diet amply supplied with methionine. The conclusion was drawn that "the effect of betaine . . . and arsenocholine are additive, and together are practically a complete substitute for choline under the conditions of these experiments" (13), and the conditions show that betaine and arsenocholine were fed together only when homocystine or methionine were also added. Hence the conclusions shown by the data with respect to betaine are that together with homocystine, or to a lesser extent with cystine, it can stimulate growth in chicks on a basal diet deficient in methionine. More recently, the statement was made (46): "For optimal gains, chicks required close to 0.06 per cent betaine-irreplaceable choline and 0.14 per cent replaceable choline in the diet." This statement applied to a diet to which homocystine had been added, and presumably should be thus restricted. Experiments with a purified diet (48) have indicated that a moderate incidence of perosis occurred in chicks on a purified diet containing 20 per cent casein and supplemented with 0.07 per cent choline, or when either 0.4 per cent methionine or 1.0 per cent betaine were added in addition to 0.07 per cent choline.

Young turkeys were fed a diet of natural foods and the effect of various supplements on perosis was studied. The incidence of perosis was lessened by adding choline or betaine to the diet. In no groups was the prevention of perosis complete. When gelatin was also added to the various diets, the incidence of perosis was increased as compared with corresponding groups receiving no gelatin. The conclusion was reached that gelatin contained a factor which inhibited choline synthesis (49). However, other explanations may be possible. For example, it has been noted that perosis occurred in turkeys as a result of niacin deficiency (50), and it has also been reported that the requirement for niacin by chicks was increased when gelatin was added to certain diets (51). The effect of various supplements on the incidence of perosis was observed in chicks on a diet of natural foods which was

supplemented with synthetic vitamins, including niacin (52). Various supplements were added, including choline, betaine, gelatin, yeast, and alcohol-extracted yeast. Perosis was observed in all groups. The addition of gelatin appeared to increase the incidence of perosis. It was speculated that the extra glycine and arginine arising from the gelatin in the diet would form glycocyamine which in turn would require labile methyl groups for the subsequent formation of creatine. This speculation would appear to require the support of extensive additional evidence, for although it is known that glycine and arginine are used in the biological formation of creatine, there is no reason to suppose that this formation, rather than oxidative processes, constitutes a pathway for the disposal of excessive amounts of glycine and arginine. It has been observed (16) that the addition of either gelatin or creatine may increase the incidence of perosis on a purified diet.

*Effects on reproduction.*—A diet was formulated to be low in choline; some of the ingredients were extracted with hot alcohol. The diet was found to contain less than .03 per cent of choline as determined by the reineckate method. Eight hens were placed on the basal diet for seven weeks and an equal number of hens in a second group were fed the same diet plus 0.2 per cent choline. During this period, no significant differences in egg production or hatchability were observed between the two groups (53). The choline content of the eggs was not determined, but if this content was assumed not to vary from values published elsewhere for normal eggs, calculations indicated that the excretion of choline in the eggs had exceeded the intake in the diet. Egg production was measured in pullets on a purified low-choline diet for an eighty-four-day period (151). Although balance-sheets were not given, it may be deduced by an approximation of the data that about twice as much choline was excreted in the yolks of the eggs as was consumed in the diet during the experimental period. Even when the casein content of the basal diet was halved, there was no marked decrease in the rate of egg production. Additions of choline had no marked effects on rate of egg production. Feed consumption and maintenance of body weight may have been improved slightly by the addition of choline to the low-protein diet.

Studies with growing turkeys on diets of natural feedstuffs indicated that the addition of choline chloride did not cause a change in weight during the first ten weeks as compared with control

groups, but the addition was accompanied by an increase in the weight of female birds at twenty-four weeks. No such increase was observed in the males. The basal diet contained 0.18 per cent of "choline" by microbiological assay (17) and the supplemented diet contained 0.23 per cent.

## Clinical Studies with Choline

*Cirrhosis.*—Observations with dogs, rats, and rabbits have indicated that cirrhosis may be developed by prolonged feeding of a diet low in choline (42, 54, 55). These observations have not unexpectedly been followed by clinical use of choline in the treatment of patients with cirrhosis of the liver. Variation has been reported in the results; this may be partly explained by the fact that cirrhosis progresses to a shrunken and fibrotic condition of the liver in which there is little possibility for a reversal towards normal.

Patients with hepatic cirrhosis were treated for more than two years with choline chloride, one gram daily, together with diets low in animal fats and cholesterol, with favorable results in a number of cases (56). However, no comparable group on an identical regimen with choline omitted was described. The effect of choline chloride on the course of ten patients with decompensated portal cirrhosis was studied (57). The patients were placed on a diet high in carbohydrate and protein and low in fat. Choline chloride was fed in doses of 1.5 to 6 gm. daily. Four of the patients died for various reasons. In the remaining six, obvious responses were noted in three cases within a week after the addition of choline and probable responses were subsequently noted in two of the others. The results indicated that the use of choline chloride may have produced some improvement in the condition beyond the effect produced by the dietary regimen. Choline alone or together with inositol was fed to four patients with cirrhosis (58). Clinical improvement was noted in all four cases. All patients received a high protein, high carbohydrate, low fat diet.

Studies with twenty-three patients with cirrhosis characterized by ascites and large palpable livers were reported (59). The patients received a high protein and low fat diet supplemented with 30 to 45 gm. of brewers' yeast daily. Eight of the patients were given choline chloride, 1.5 to 3 gm. daily, together with an equal amount of cystine, for one to five months. The remaining fifteen patients did not receive choline or cystine. Only one member of the treated group was dead at the end of one year as compared

with eight deaths in one year in the untreated group. Signs of clinical improvement included elimination of the ascites, and increase in the serum protein levels. A third group of twelve cirrhotic patients whose livers were not enlarged showed no response to the therapy.

It was reported (157) that choline chloride, 10 gm. to 25 gm. daily, was given for varying periods to three patients presumably suffering from portal cirrhosis without producing a demonstrable effect upon the clinical course of the disease or on the serum lipids. Another patient received 1 gm. daily, and cholesterol and lipid phosphorus fell while under this treatment.

The complex nature of cirrhosis, and its grave prognosis, make it difficult to accumulate clear-cut data with respect to the ameliorative effect of any dietary ingredient, but results to date indicate that supplementation with choline is a desirable adjunct to supportive treatment.

*Choline and methionine in the treatment of infectious hepatitis.*—Methionine in doses of 10 gm. was administered intravenously to patients with infectious hepatitis with an apparently beneficial effect. In a case described as typical there was a fall in serum bilirubin within twenty-four hours, improvement in appetite, and a marked gain in weight starting four days later. The authors indicated that the chances of a response to methionine were best when the hepatitis is uncomplicated by cirrhosis or extensive cellular destruction (60). In contrast, methionine in a dosage of 5 gm. daily was found to be ineffective in altering the course of infectious hepatitis in a large series of adequately controlled cases (61, 62). The course of infectious hepatitis was studied (63) by several criteria including the length of time to reach the first day of fall of serum bilirubin to 0.6 mg. per 100 cc., the first day of bile-free urine and the first day of return of appetite. Choline chloride, 1.5 gm. daily, was administered as a supplement to a low fat, high carbohydrate diet with the addition of vitamins and was found to be ineffective in shortening the course of the disease by comparison with a group of patients who received the same regimen without the administration of choline. It was suggested, however (64) that the dosage used by some workers in the treatment of infectious hepatitis was insufficient and a dose of 20 gm. of choline chloride daily for two or three weeks or 20 to 60 gm. of methionine daily for an indefinite period was suggested. It was stated that the use of doses of this size usually led to ketosis, the ketones being

acetone and β-hydroxy-butyric acid in the case of methionine and an unidentified ketone or aldehyde in the case of choline. Details of the effect of the treatment on the course of the disease were not described. Owing to the acetylcholine-like effect of choline when injected, the use of such high dosage appears to involve a risk of side reactions. No acceleration of recovery from acute infectious hepatitis was observed in patients who received choline chloride, 5 gm. daily for ten days, or methionine (158). The preponderance of the evidence suggests that no definite role can as yet be assigned to choline and methionine in the treatment of the disordered functions of the liver which accompany infectious hepatitis. The lack of unanimity among investigators indicates a need for further research in this field.

*Choline and anemia.*—Choline chloride in doses of 10 mg. per kg. of body weight orally three times daily to three adult human males for ninety days failed to produce either anemia or macrocytosis (65). A patient with macrocytic anemia and cirrhosis of the liver received choline chloride, 17 mg. per kg. of body weight, daily for seventy days (66). He was maintained on the regular hospital diet and received no other medication. During the period of choline treatment, the red blood cell and hemoglobin content of the blood progressively increased. Choline was administered intravenously, 1 gm. daily, to a patient with pernicious anemia who had failed to respond to purified liver extract (67). Prior to treatment, the patient showed fatty metamorphosis of the liver and bone marrow. The anemia responded with a reticulocyte peak of 6 per cent in three days and steady increases in hemoglobin and erythrocyte count. Following treatment, the fat content of the bone marrow decreased to approximately one-tenth of the initial value. It was concluded that the results indicated that an adequate amount of the antipernicious-anemia factor was "stored in the liver but not effectively elaborated because of the fatty state."

## Lipotropic Factors

*Choline in the nutrition of the depancreatized dog.*—The original observations regarding the dietary production and prevention of fatty livers were made with depancreatized dogs (68, 69). When kept on a diet of lean meat and sugar and maintained with insulin, these animals developed fatty livers (69) which were prevented by the addition of fresh beef pancreas to the diet (70). The factor in

pancreas responsible for the prevention was postulated to be lecithin, and crude preparations of this substance were found to prevent the fatty livers (71, 72, 73). Following this, the attention of the Toronto group was changed to observations with the normal rat as an experimental animal (73, 74, 75). Paradoxically enough, and although lecithin and choline are effective in preventing fatty livers in depancreatized dogs (76), it now appears that there is a major active factor in raw pancreas under the conditions of such experiments (69 to 73) which is not lecithin or choline but is probably an enzyme which releases methionine from protein combination (29), although raw pancreas in large daily feedings of 250 gm. supplies enough choline to prevent fatty livers (77). The freed methionine is then presumably available for conversion to choline; methionine was shown in other investigations (28) to replace choline in preventing an acute nutritional deficiency in young dogs. The fatty liver syndrome (29) appeared in depancreatized dogs receiving in their diet 500 gm. of lean meat daily, which supplied 0.5 gm. of choline and 3 gm. of combined methionine. The syndrome was alleviated either by feeding additional choline, or by feeding free methionine, or by feeding a small quantity of a fraction concentrated from fresh pancreas. The function of this fraction was presumably the liberation of methionine into the free state from the meat present in the diet.

The concentration of this fraction, the "antifatty liver factor" has been made possible only by a time-consuming series of experiments made with depancreatized dogs. The factor was shown to be present in fresh pancreatic juice (78). This material was shown to raise the cholesterol, phospholipid and total fatty acid content of the blood of depancreatized dogs, in addition to preventing fatty livers (79). The factor was present in dried acetone-and-ether extracted pancreas, and it was replaceable by choline although the activity of the pancreas fraction was not explainable in terms of its choline content (77). More potent concentrates were obtained by extracting pancreatic tissue with dilute acid, precipitating the extract with ammonium sulfate, dialyzing, and finally precipitating with acetone. The final preparation was free from choline and was active at a level of about 6 mg. per kg. of body weight per day in the prevention of fatty livers in the depancreatized dog maintained with insulin and on a lean meat diet (80). The daily choline requirement as a substitute for the pancreas fraction in the prevention of fatty livers under these conditions was about 35 mg. per kg. of body weight per day; 15 mg.

was insufficient (77). Under the same experimental conditions, *dl*-methionine, 260 mg. per kg. of body weight daily, was an effective substitute for choline or pancreas in the prevention of fatty livers in depancreatized dogs. The concentration of choline in the blood plasma of these dogs (81) was found to be reduced to values of 20.6 to 45.0 mg. per 100 cc. at twelve to fifty-six weeks following pancreatectomy. When an active fraction of pancreas was fed, the plasma choline level rose to levels of 61.9 to 77.5 mg. per 100 cc. which were at least as great as the preoperative levels in a comparable series of dogs. The plasma choline was found to be present almost entirely in the form of phospholipids (82).

It appears from the above series of investigations that in dogs, deprivation of the external secretion of the pancreas results in an increased dietary requirement for choline. This increased requirement is presumably due to an interference with the supply of methionine which is normally liberated from proteins in a utilizable form by the action of an enzyme present in pancreatic juice. The possibility that methionine might be needed in the diet of these dogs for physiological functions in addition to serving as a precursor of choline is not excluded, although such a possibility is not evident in the experimental data.

*"Lipocaic."*—The name "lipocaic" was applied to a fraction from pancreas which prevented or relieved fatty livers in depancreatized dogs (83). Originally the fraction was prepared as follows: Beef pancreas was mixed with two volumes of 95 per cent ethanol, the ethanol was strained off and the extraction was repeated twice. The combined extracts were evaporated to dryness and fatty materials were removed by extraction with ether (83). The preparation of active fractions was more recently carried out by extracting raw beef pancreas with 95 per cent alcohol, acidified with sulfuric acid to pH 3. The final concentration of alcohol was 60 per cent. This was followed by neutralizing with calcium hydroxide, filtering and drying (84). Methionine, 0.5 per cent in the diet, or choline, could replace the pancreas fraction in preventing fatty livers in rats fed a high fat, low protein diet, but the fraction contained so small an amount of methionine, 1.25 to 1.28 per cent, that this substance could not account for the biological activity. The preparation was found to contain "no choline" which eliminated the possibility of choline being responsible for the activity of the pancreas fraction. At a daily level of 136 to 160 mg., a "lipocaic" preparation (filtered pancreas extract) was effective when fed and was also effective when injected, preventing fatty

livers in rats (85). Inositol was ineffective at a level of 1.6 per cent in the diet. The results with the pancreas extract were taken to indicate that the active principle in such extracts did not need to pass into the alimentary tract to exert its effect. This assumption seems rather broad, but it is quite possible that the enzymatic fraction discussed on page 206 (81) might not exert its postulated effect on dietary protein if administered by injection into intact rats; indeed, there is abundant evidence that rats under similar conditions are able to utilize casein for the prevention of fatty livers without supplementation with any form of pancreatic extract (86). The complete absence of choline from the pancreatic extract (84) may be contrasted with the relatively large amounts of choline, 1.0 to 2.45 per cent, found (87) in a preparation resulting from extraction of pancreatic tissue with 40 to 60 per cent alcohol. It was reported, however, that the choline content of the latter preparation accounted for only one-third of its antifatty liver effect in rats. In another investigation (88) an alcoholic extract of beef pancreas was found to contain 0.5 per cent of choline and to be relatively effective in preventing hemorrhagic kidneys and fatty livers in rats when fed at a level of 1 per cent in the diet. Its protective effect appeared to be greater than could be attributed to its choline content.

*Lipotropic activities of various substances.*—N′-methylnicotinamide, with or without added homocystine, was found to have no lipotropic activity at a level of 1 per cent when fed to rats which received a diet deficient in labile methyl groups (89). However, prevention of fatty livers was reported to occur as a result of feeding N′-methylnicotinamide at a level of 2 per cent to rats on a low-labile-methyl diet plus glycocyamine (90). It was also stated that the administration of N′-methylnicotinamide increased the urinary excretion of nicotinic acid. These observations were perhaps unexpected since it had been previously reported that there was no increase in the urinary excretion of nicotinic acid following the administration of N′-methylnicotinamide to human subjects (91). Similarly a simultaneous increase in liver fat and in urinary N′-methylnicotinamide excretion had been previously described in rats which were fed nicotinamide (92) which would indicate that N′-methylnicotinamide was formed at the expense of lipotropic substances rather than contributing to their formation.

Difficult to interpret in terms of the present knowledge of choline and labile methyl are the observations that atabrine at a

level of 65 mg. per 100 gm. of diet (93) and dimethylaminoazobenzene (94) were effective in preventing hemorrhagic kidneys in rats on a labile-methyl deficient diet. Unilateral nephrectomy caused an increase in the susceptibility of adult rats to the hemorrhagic kidney syndrome during the period immediately following the operation, but if a postoperative period of two weeks was allowed to elapse before placing the rats on the deficient diet, the incidence of renal lesions was low (88).

The lipotropic action of inositol was found to be abolished by adding corn oil, 1 per cent, to a basal low-choline diet. The lipotropic effect of choline was undiminished under similar conditions (95).

*The "biotin fatty liver."*—Rats were fed a diet containing only 10 per cent of casein and with the entire vitamin B complex omitted. After three weeks, various vitamin supplements were added. The inclusion of biotin concentrates and, in one experiment, crystalline biotin, resulted in the rats showing improved growth and increased fat and cholesterol content of the liver as compared with rats not receiving biotin (96). The term "biotin fatty liver" was applied to the phenomenon observed in these experiments, and it was stated that the condition was resistant to the action of choline but could be prevented by inositol (32). These statements were challenged (97) in a reinvestigation of the problem. In this communication confirmation was obtained of the observation that the administration of a biotin concentrate tended to increase the liver fat and cholesterol content when administered to rats which received certain B vitamins following a depletion period as compared with controls which received the same experimental treatment except that biotin was omitted. If, however, B vitamins were fed during the fore period, no significant effect was obtained from biotin. In all the experiments in this investigation, choline was effective in markedly reducing the fat and cholesterol content of the livers. Inositol had a slight lipotropic effect. The abandonment of the term "biotin fatty liver" was recommended. In another investigation (98) rats were placed for three weeks on a depletion diet similar to that described above (96) and were then fed various vitamin supplements with or without choline and inositol for one week, following which the fat content of their livers was measured. It was noted that on certain supplements the liver fat became further decreased if the feeding period with choline was extended to twenty-four days. The conclusion was reached that "fatty livers

due to choline deficiency are not found in animals which are, for any reason, losing weight and that the extent of the accumulation of the liver fat in choline deficiency is roughly proportional to the food consumption and growth rate . . . ." This conclusion appears valid from consideration of the data presented. The further statement was made (98) that "there is no real justification for the use of the term 'biotin fatty liver'. " The investigation is of interest in that the use of a sulfonamide in the diet was introduced. This might be advantageous in investigations in which a role is sought for biotin in the etiology of fatty livers, bearing in mind the demonstration that the signs of biotin deficiency in the rat are not manifested on purified diets unless raw egg white or a sulfonamide are added (99). The "biotin fatty liver" appears to be a condition which has been observed only in rats which received a special dietary treatment including a preliminary subjection to a multiple vitamin deficiency.

## Miscellaneous Observations

*Choline in the nutrition of* Neurospora crassa.—A mutant, No. 34486, of *Neurospora crassa* was found to need choline for growth in a medium of sugar, salts and biotin (17). The mutant did not respond to betaine, and methionine had only a slight effect. The use of the mutant was proposed as an assay method for choline in natural materials. The method included the use of permutit for the separation of choline from methionine. Applications of the procedure to various natural materials were described (100, 101, 102). It was shown later, however, that the method was not specific for choline since a similar response was obtained with dimethylaminoethanol (22) and a less marked response with methylaminoethanol (20). These and other observations (18) were made the basis of a suggestion that the chick and *Neurospora* mutant No. 34486 were unable to carry out the reaction shown in Fig. 1, step 6, but were able to carry out the reactions shown in steps 7 and 8 in the formation of choline (18, 20). Confirmation of the growth-promoting effects of methylaminoethanol and dimethylaminoethanol on mutant No. 34486 was reported (21) and this mutant was found to respond also to arsenocholine, calcium phosphoryl-choline chloride, dimethylethylhydroxyethylammonium chloride, and less markedly, to methyldiethylhydroxyethylammonium chloride and triethylhydroxyethylammonium chloride. A second mutant, No. 47904, behaved similarly except that meth-

ylaminoethanol was less effective for this mutant than for No. 34486. It was noted that a substance which showed activity for both mutants accumulated during growth of mutant No. 47904. Tests led to the conclusion that the substance was not methylaminoethanol. However, in a later publication (103) this conclusion was stated to be erroneous, and the isolation of methylaminoethanol from cultures of the last-named mutant was described. An aqueous extract of liver was precipitated with reineckate at pH 8 to 9 to remove choline (20). It was found that the filtrate was active for mutant 34486, which might have indicated the presence of methylaminoethanol or dimethylaminoethanol. Attention was drawn (104) to the observation that higher values for choline were obtained with the *Neurospora* assay (17) than with the reineckate procedure (105) for the determination of choline in certain natural foods. These results supported the view that precursors of choline might exist in a simplified diet which contained corn and peanut meal.

*Choline and anemia in dogs.*—Reduction of the erythrocyte count was reported to occur in dogs following the feeding of choline chloride at the remarkably small dosage rate of 8 mg. per kg. of body weight per day (106). The daily feeding of 5 gm. of soybean "lecithin" per dog had a similar effect. Atropine was reported, on the basis of observations with two dogs, to block the effect of choline. The feeding of lard, 60 gm. daily, with choline, 10 mg. per kg. of body weight daily, to four normal dogs was stated to cause reductions in erythrocyte counts and hemoglobin percentages. The fat was postulated as furnishing hemolytic agents which increased red cell destruction (107). In a further communication (108) it was reported that five dogs became anemic while receiving choline chloride by stomach tube. The dosage was 10 mg. per kg. of body weight, fed at first once daily, then increased to twice and later to three times daily. The animals were maintained on a diet of commercial dog food and rolled oats. In one dog the anemia responded to atropine; in another dog it responded to dried stomach, and in the other three dogs to the injection of liver extract. Later (109) it was found that eight dogs became anemic while receiving acetylcholine by injection or choline by stomach tube. Pteroylglutamic acid produced a remission of the anemia in three of the dogs and liver extract produced remission in three others. Observations made with the blood serum led to the conclusion that the action of either pteroylglutamic acid or

liver extract in the treatment of anemia probably is to increase the cholinesterase activity in the body. This series of investigations awaits confirmation. The administration of choline did not produce anemia in human subjects (67). In dogs, it has been shown that the daily choline requirement following pancreatectomy is about 35 mg. per kg. of body weight per day; 15 mg. was insufficient (77). This may be compared with the dosage of 8 mg. per kg. reported (109) to produce anemia. The amount of choline, pteroylglutamic acid and perhaps other factors in the diet (108) is a matter of conjecture.

Hemorrhagic lesions were described in the nervous system of dogs which had received small doses of choline chloride, physostigmine and carbamyl choline (110).

*Toxic levels of choline.*—Further studies were made with rats (111). Solutions of choline chloride fed by stomach tube showed an increase in toxicity when the concentration of the solutions was increased. The 50 per cent lethal dose ranged from 3.4 to 6.1 gm. per kg. of body weight. Respiratory paralysis was noted together with the excretion of a protoporphyrin-containing discharge from the Harderian glands. This discharge has previously been described as a result of the administration of choline and in certain nutritional deficiencies; the earlier work has been reviewed (112).

The toxicity of choline in the diet of growing chicks (113) was measured by adding choline chloride at levels of 1, 2 and 4 per cent to rations of a "general starter" type. The rate of gain in body weight was depressed by about 12, 13.8, and 23.8 per cent respectively on the diets containing the three levels of added choline. Other than the reduction in growth rate, no gross pathological lesions were observed. Of interest was a decrease in the body fat deposits which became more marked as the level of choline was increased.

It was reported that a pathological condition was observed in cows, the signs included a failure of the uterus to involute after calving (114). It was simultaneously noted that brewers' grains formed a considerable part of the ration and that choline could be isolated from an extract of brewers' grains. Although the logic of the assumption does not seem obvious, it was concluded from these observations that choline was responsible for the pathological condition.

*Phosphorylated choline compounds.*—The distribution of labeled phosphorus was studied in the tissues of rats following the

injection of phosphorylcholine which had been synthesized with $P^{32}$ in the molecule. The ester appeared to be converted to inorganic phosphate with great rapidity by the body and it did not enter the phospholipids as a unit. There was some indication that the administration of phosphoryl choline slowed down the formation of phospholipid in the liver (115).

Beef pancreas was macerated, mixed with water, and incubated at 37° for four hours. α-Glycerophosphorylcholine was isolated from an extract of the autolyzate. Balance sheets indicated that the substance was formed by the enzymatic hydrolysis of lecithin. The action of "lecitholipases" was stated to be responsible for the formation of the glycerophosphorylcholine from lecithin; pancreatic lipase did not split lecithin (116). The formation of a similar compound from lecithin during the autolysis of rat stomach and intestine was described (117). Substances resembling choline glycerophosphates were noted in acid extracts of dried beef pancreas (118).

*Determination of choline.*—On the basis of a careful study of the application of the reneckate method for the estimation of choline in natural materials (119), certain recommendations were made, including hydrolysis with barium hydroxide for ninety minutes, neutralization to thymolphthalein (pH 8 to 9) with acetic acid, washing the reineckate precipitate with *n*-propanol, and measuring the absorption of the reineckate, dissolved in acetone, at 526 mμ. These modifications were found to avoid the precipitation of betaine reineckate, which was insoluble in acid solutions but soluble in slightly alkaline solutions. However, it has recently been noted (20) that dimethylaminoethanol appears to be partly carried down in the choline reineckate precipitate when a solution containing a mixture of choline and dimethylaminoethanol is treated with reineckate at pH 8 to 9, although dimethylaminoethanol in pure solutions is not precipitated by reineckate in this pH range.

The above method (119) was compared (82) with a method in which the reineckates were precipitated from the barium hydroxide hydrolysate in an acid medium (120). The methods were in agreement when applied to an alcohol-ether extract of blood plasma or to a fraction precipitated from a petroleum-ether solution of an alcohol-ether extract of liver by acetone and magnesium chloride. The untreated alcohol-ether extract of liver gave higher values when analyzed by the second method (120) than by the

first method. It was concluded that the precipitation step served to rid the phospholipid fraction of compounds that formed acid-insoluble reineckates.

The use of ultraviolet absorption at 327m$\mu$ in measuring choline reineckate was reported to increase the sensitivity of the reineckate method very markedly (121).

*Phospholipid turnover.*—Further studies were made (122) in the use of radioactive phosphorus in following phospholipid metabolism. Dogs were injected intravenously with radioactive disodium phosphate in isotonic solution. Some of the dogs received large doses of choline chloride a short time before the phosphate, others received no choline and served as controls. The specific activity of the choline-containing phospholipid fraction of the liver increased far more greatly in six hours in the choline-treated dogs than in the controls. The choline-treated dogs also showed a simultaneous increase in the specific activity of the choline-containing phospholipids of the plasma; the increase was greater than that observed for the controls, but was less than the increase in the activity of choline-containing phospholipids of the livers of treated dogs. It had been shown elsewhere (82) that the phospholipids of plasma were predominantly choline-containing. These observations supported the conclusion that the phospholipids of plasma are formed in the liver. It was found that excluding the liver from the circulation resulted in a marked slowing of the rate of plasma phospholipid turnover (152). This observation indicated that the liver was concerned with the removal of the plasma phospholipids as well as with their synthesis.

The level of phospholipids in the livers of rats on high fat diets was found to be increased by supplementing the diets with choline. On low fat diets, the levels were only slightly affected by adding choline (123).

A small but definite amount of labile methyl was synthesized by rats on an adequate diet. This was demonstrated by observing the presence of deuterium in the methyl groups of choline isolated from rats which had received drinking water containing deuterium oxide for three weeks (124). The action of intestinal bacteria was one of the explanations which could account for the synthesis.

*Excretion of choline.*—When large doses of choline chloride were fed to sheep and dogs, only 0.5 to 2.5 per cent of the amount of choline injected was recovered in the twenty-four-hour urine. In sheep, the levels of free and total choline in the liver, kidney or

plasma were not increased by feeding choline (125). Similarly low proportions, 0.7 to 1.5 per cent, of the choline intake were excreted by human subjects (126). The total output of choline in urine plus feces plus sweat was measured. Only 0.01 per cent of the intake was excreted in the sweat even under "hot moist" conditions. No increase in total choline excretion was found under "hot moist" conditions.

*Protective actions of choline.*—Studies of the effect of diet on the toxicity of ethylene dichloride were described (127). A basal diet was used which was low in choline. Ethylene dichloride was administered by inhalation and it was found to cause high mortality. The susceptibility was increased by lowering the protein and increasing the fat content of the diet. Adrenal cortical lesions were described in some of the exposed rats. The mortality after exposure was greatly reduced by supplementing the diet with methionine and choline or by increasing the casein content of the diet; the addition of methionine and choline to the low protein diet did not satisfy the amino acid requirements for growth but this addition had a protective effect equal to that of the high casein diet. The incidence of hemorrhagic necrosis of the adrenal cortex was greatest in rats fed low-casein high-fat diets and did not occur with control diets.

Administration of a labile-methyl deficient diet plus alcohol was found to produce cirrhosis of the liver in rats in sixty-three to eighty-four days. The cirrhotic process was checked and the histological appearance of the parenchyma was markedly improved when choline chloride was fed at the rate of 40 mg. daily or when the casein content of the diet was raised to 30 per cent (128).

A most interesting report (155) described the occurrence and nature of neoplasms in the liver, lungs and other tissues of rats as a result of prolonged choline deficiency. The diet was supplemented with minimum doses of choline to prevent death from acute deficiency. Neoplasms were observed in twenty-nine of fifty deficient animals. No lesions were observed in the litter-mate controls which received 20 mg. of choline daily. Similar results (156) were observed with a somewhat different basal diet. Neoplasms were observed in sixteen of twenty animals at seven to eleven months. No spontaneous neoplasms were observed in twenty control rats which received the same basal diet plus 0.2 per cent choline chloride.

A brief communication indicated that choline chloride showed

indications of a protective effect against experimental yellow fever in rhesus monkeys. Five of eight monkeys which received choline chloride survived after inoculation with a potent yellow fever virus which had shown uniformly lethal properties in previous experiments. The liver pathology in the control monkeys appeared more extensive than in the monkeys which received choline (147).

Methylaminoethanol and dimethylaminoethanol were found to improve the work performance of the failing mammalian heart. Heart-lung preparations of dogs were used. The action of these substances was manifested without first atropinizing the heart preparation; in this respect the similar action of choline was not shown until atropine had been administered (129).

The effect of various dietary factors was studied on the deposition of vitamin A in the livers of rats. On a high-fat low-protein diet, the amount of vitamin A stored was not affected by the presence of excess liver fat or by choline deficiency. With a low-fat low-protein basal diet, the addition of choline increased the storage of vitamin A somewhat (130).

*Choline acetylase.*—This subject was recently reviewed (153). The enzyme system (131) formed acetylcholine under anaerobic conditions in the presence of choline, acetate, and adenosinetriphosphate. Potassium was required for the action of the enzyme; magnesium, manganese, cysteine and glutamic acid also had an activating effect. The enzyme preparation consisted of the supernatant liquid from a buffered homogenate of rat brain tissue. Incubation was carried out for fifteen minutes at 37° under nitrogen in Warburg vessels. Further activation was obtained with an unidentified coenzyme (132) which was present in various tissues and was concentrated by precipitation with barium. The coenzyme was concentrated (133) a hundredfold; it is possibly a nucleotide, and the enzyme system appeared to be similar in all respects examined to the one which condenses sulfanilamide and acetate. Indications that the same coenzyme was concerned with phosphorylation of acetate in pigeon liver were noted (134). The presence of the enzyme in the optic nerve of the rabbit (132) was thought to be of importance in considering the possible role of acetylcholine in the conduction of nerve impulses. Diisopropyl fluorophosphate which causes prolonged or irreversible inactivation of cholinesterase (135), did not affect choline acetylase. Formation of acetylcholine by choline acetylase in the nerve axon was observed (136) in a preparation of rabbit sciatic nerve. The rate of formation was

markedly diminished seventy-two hours after section of the nerve.

The activity of the enzyme system which synthesized acetylcholine in nervous tissue was increased by adenosinetriphosphate, citrate, and an unidentified activator obtained from brain tissue. The inactive dialyzed enzyme was not reactivated by any of the above three factors but was by citrate and activator and especially by all three. The enzyme was prepared from a saline extract of brain tissue (137). In another study of the enzyme system (154), experiments were described which indicated that at least five components were required for maximum activity; choline, potassium, adenosinetriphosphate, a coenzyme present in boiled aqueous extracts of brewers' yeast or animal tissues, and a substrate which could be citrate, acetoacetate or *cis*-aconitate. The possible formation of "active" acetate from the substrate as a preliminary step in the acetylation of choline was discussed.

Studies of the effect of various added substances on the aerobic formation of acetylcholine by frog brain tissue under aerobic conditions were continued (138). The substances were added to a mixture of frog brain, physostigmine, glucose and Ringer's solution and the mixture was incubated for four hours. An increase in acetylcholine formation was observed when $\alpha$-tocopherol was added, and the possibility that vitamin E is a part of the coenzyme involved in the synthesis of acetylcholine was suggested. An antioxidant effect is perhaps a second possibility in view of the anaerobic activity of choline acetylase (131). A large number of other substances caused lesser modifications of the yield of acetylcholine in such tissue preparations (139).

*Other observations.*—Competition between choline and triethyl choline in the choline-acetylcholine system was investigated (140). Triethyl choline was found to block the response of the frog gastrocnemius to choline, but it did not block the response to acetyl choline. This could be explained by assuming that the triethyl compound interfered with the acetylation of choline but did not interfere with the action of acetylcholine on the muscle. It was also noted that choline protected against the toxic action of triethyl choline when injected into mice.

Methionine sulfoxide was reported to be capable of acting as a methyl donor in a rat muscle preparation with the formation of choline and creatine when ethanolamine and glycocyamine were respectively used as substrates (141).

Local application of choline chloride was reported to cause the

disappearance of the fatty substances in pterygium (142).

Studies of the blood choline level of young calves (143) indicated a rise during the first three weeks when the calves received milk. Removal of milk from the diet at thirty-five days was accompanied by a decrease in the blood choline level. Colostrum milk was found to be higher in choline than later milkings.

When rats were fed choline chloride, 0.2 to 0.5 mg., in addition to 1 cc. of olive oil, the movement of fat into the areolar tissue of the villus and into the cells of the small intestine appeared to be more rapid than when the rats were fed olive oil without choline chloride (144). Radioactive phosphorus was used in an investigation of the rate of accumulation of phospholipids in the intestinal mucosa (145). It was found that the feeding of choline caused an increase in the radioactivity of the intestinal phospholipids, especially when fat was simultaneously ingested.

Choline was observed to be necessary for the formation of histiocytic cells; betaine was ineffective (146).

Bronzed hair pigmentation was noted to develop in rats which received a purified diet containing a high level of choline chloride (148).

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# THE CHEMISTRY OF THE PROTEINS AND AMINO ACIDS*

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## AMINO ACIDS AND PEPTIDES

Nearly complete data on the amino acid composition of a number of proteins have been reported by Chibnall in his Proctor lecture [(1), cf.(2)] and by Brand [(3), cf.(4) and table III]. A detailed account of the work carried out in Chibnall's laboratory is available in publications by Bailey *et al.* (5) and by Chibnall *et al.* (6) on the determination of the dicarboxylic amino acids, by MacPherson (7) on the basic amino acids, by Rees (8) on serine, threonine, "periodate ammonia" and amide ammonia, by Tristram (9) on the partition chromatography of monoamino acids and by Gale & Epps (10, 11, 12) on the use of specific decarboxylases.

*Reporting of data on the amino acid composition of proteins.*—With the accumulation of extensive data on the amino acid content of proteins and foodstuffs, problems have arisen as to the most appropriate way of reporting this information. For pure proteins Brand (3, 4) prefers to report his results as gm. of amino acid per 100 gm. of protein (dry, sulfate- and ash-free), as gm. of amino acid residue (anhydride) per 100 gm. of protein, as gm. of amino acid nitrogen per 100 gm. of protein, as moles of amino acid per $10^5$ gm. of protein and as residues of amino acid per mole of protein. In accounting for the split products of a protein as amino acid residues a value for "Terminal $H_2O$" has to be added for those amino acids that are not in peptide linkage and presumably terminal. This value for "Terminal $H_2O$" corresponds to the free (terminal) $\alpha$-amino nitrogen, *i.e.*, one hydrogen atom in the terminal $\alpha$-amino groups and one hydroxyl group in the terminal car-

* This review covers the period from October 1945 to December 1946.

boxyl groups (3, 4). In the calculation of the results as residues, the amide ammonia is arbitrarily assigned to either glutamine or asparagine, or both, since it is not yet possible to differentiate between these two amides. If a protein is composed only of amino acids in peptide linkage, the sum of the split products on a residue basis will be 100 gm. per 100 gm. of protein, while this sum calculated as amino acids will be larger, usually about 118 gm. per 100 gm. of protein. The difference between these two figures is the water taken up on hydrolysis, usually between 17 and 18 gm. per 100 gm. of protein. If a protein contains carbohydrate in addition to amino acids, the sum of the amino acid split products calculated on a residue basis will amount to less than 100 gm. per 100 gm. of the protein, although its nitrogen has been fully accounted for.

Since it is now possible to determine the amino acid composition of proteins, it will become imperative to find a compact way of presenting the empirical formula of a protein in terms of its amino acid residues. It has been proposed (3, 4) to use as symbols, whenever feasible, the first three letters of the amino acids: *e.g.*, glycine residue (Gly); isoleucine residue (Ileu), etc. The residues of cysteine, half-cystine, asparagine and glutamine are designated by (CySH), (CyS-), (Asp-$NH_2$), and (Glu-$NH_2$), respectively; "terminal $H_2O$" by ($H_2O$).

Symbols for Amino Acid Residues (3, 4)

| | | |
|---|---|---|
| Gly | (CySH) | Glu |
| Ala | (CyS-) | (Glu-$NH_2$) |
| Val | Met | (Asp-$NH_2$) |
| Nval | Try | Ser |
| Leu | Arg | Thr |
| Ileu | His | Tyr |
| Pro | Lys | Hypro |
| Phe | Asp | Hylys |

From the empirical formula of crystalline pepsin (13), shown below, the detailed amino acid composition, the nitrogen and sulfur content, the number of residues per mole, etc., can be calculated. The molecular weight was calculated from the phosphorus content; the alanine content was not determined (13).

PEPSIN, MW = 34,400, Total N = 14.60%

$Gly_{29}$ $Ala_{..}$ $Val_{21}$ $Leu_{27}$ $Ileu_{28}$ $Pro_{15}$ $Phe_{9}$ $(CySH)_2$
$(CyS\text{-})_4$ $Met_4$ $Try_4$ $Arg_2$ $His_2$ $Lys_2$ $Asp_9$ $Glu_{28}$ $(Asp\text{-}NH_2)_{32}$
$Ser_{40}$ $Thr_{28}$ $Tyr_{16}$ $(H_2O)_2$ $(H_2PO_3)$

Martin & Synge (14) and Chibnall (1) believe that it would be a great advance if the data for each amino acid were expressed in the form of nitrogen as a percentage of the total protein-nitrogen. Chibnall (1) contends that such recording of the results permits one to see at a glance how close the summation is to one hundred. The authors of this review feel that such reporting is valuable as an additional feature, but that the reporting of data as gm. amino acid per 100 gm. of protein (and when indicated also as gm. amino acid residue) is fundamental and should not be omitted. This is particularly important because many proteins contain constituents other than amino acids such as carbohydrates, glucosamine, fatty acids and others. One recent publication (8) from the Biochemical Laboratory at Cambridge is very satisfactory, because all the data have been recorded both ways.

An entirely different problem arises in connection with the reporting of the amino acid composition of foodstuffs and other protein containing materials. In a monograph by Block & Bolling (15) the results of amino acid determination are computed upon the uniform basis of a hypothetical substance that contains 16 per cent nitrogen. Comprehensive tables of data are presented, in all of which the original figures from the literature have been recalculated on this basis. Gelatin, edestin, crystalline egg albumin, β-lactoglobulin, insulin, hemoglobin, etc., have been treated the same way as tankage, meat scraps, grass, flour, etc. This procedure of calculating the amino acid content on the basis of 16 per cent of nitrogen has been severely criticized by Vickery & Clarke (16). With this criticism the authors of this review agree, particularly as far as proteins are concerned. Hart (17), from the point of view of the nutritionist, suggests that the amino acid content of a food or feed material be expressed either (*a*) as per cent of the dry material, or (*b*) as per cent of the dry, fat-free material, or (*c*) as per cent of the total nitrogen either on a fat and water-free basis or as the material occurs. Hart (17) points out further that the procedure under (*c*) still lacks absolute accuracy due to the fact that not all of the nitrogen of a food material is of known nutritive value (nor is it all protein nitrogen), but that the method is not clouded by hypothetical conversion factors. One of the difficulties in the situation is that at present we are able to determine the amino acid content of food materials with greater accuracy than their content of protein.

*Determination of amide nitrogen.*—It has been considered as well established that the ammonia present in acid hydrolysates of proteins is derived from two sources: (*a*) from the liberation of ammonia from the acid amides, asparagine and glutamine; and (*b*) from the decomposition of amino acids or peptides. The amino acids that are decomposed to a considerable extent during the usual acid hydrolysis (e.g., with 6 *N* HCl for sixteen to twenty-four hours) are serine (1, 3, 4, 8) and threonine (1, 8); to a lesser extent, tyrosine (8), cystine (8) and leucine (8, 18). While the ammonia is very easily liberated from glutamine [cf. (19)], asparagine is more resistant to hydrolysis.

The methods for the determination of the the true amide ammonia were, therefore, based upon a mild hydrolysis. In order to minimize ammonia formation from decomposition of other amino acids in all these methods, it was assumed that the mild hydrolysis would set free the amide ammonia groups first and that the destruction of amino acids would proceed over a long period of time at a uniform rate so that an extrapolation to zero time would yield the true amide ammonia value. This is admittedly not an entirely satisfactory procedure and values for the amide ammonia cannot be looked upon with full confidence. As yet, there are no satisfactory methods to differentiate between the glutamine and asparagine.

The method for the determination of amide ammonia devised by Gordon *et al.* (20) (hydrolysis at 37° with concentrated hydrochloric acid) was applied to a considerable number of proteins in Chibnall's laboratory (1, 2). The detailed protocols of these experiments have now been reported by Rees (8). He determined in a number of proteins both the amide ammonia and the ammonia formed during total hydrolysis; at the same time, amino acid mixtures corresponding to certain proteins were similarly treated. Rees could show that the ammonia formed during total hydrolysis was not fully accounted for by amide ammonia and that formed from the decomposition of serine and threonine. Particularly great was the discrepancy in the case of gelatin, insulin and $\beta$-lactoglobulin. Some of this extra ammonia is, of course, due to the destruction of amino acids other than serine and threonine, but part of it may be due to a greater sensitivity of hydroxyamino peptides. Warner & Cannan (21) have used a mild alkaline hydrolysis for the determination of amide ammonia; and Saidel & Brand [(22) cf. (3, 4)]

have adapted their procedure to Conway vessels. Since the amide nitrogen plays an important part in the nitrogen partition of proteins, the development of more specific and more accurate methods for the determination of this constituent seems highly desirable.

*Determination of free amino nitrogen.*—This determination is carried out on the intact protein in the Van Slyke apparatus; preferably the manometric apparatus is used. It is advisable to control the temperature during the determination by letting water of a desired temperature flow at a rapid rate through the chamber of the apparatus (4). The total free amino nitrogen measured in the Van Slyke apparatus is assumed to be derived from (*a*) the $\epsilon$-amino groups of the lysine residues and (*b*) from the free $\alpha$-amino groups of the terminal amino acids after a reaction time of about thirty minutes (1 to 4). This is somewhat arbitrary, since nitrogen continues to be developed after thirty minutes. The accessibility of the lysine chains to deamination is not the same in different proteins, but varies markedly from protein to protein (4). It is, therefore, not possible to differentiate in the manometric Van Slyke apparatus between nitrogen contributed by free $\alpha$-amino and $\epsilon$-amino groups of proteins. Computations for these two types of nitrogen on the basis of the reactivity of the $\epsilon$-amino nitrogen of free lysine do not seem to be permissible [(4), cf. (2)]. The free $\alpha$-amino nitrogen of a protein can be obtained only by deducting the $\epsilon$-amino nitrogen (derived from a determination of the lysine content) from the total amino nitrogen found in the Van Slyke apparatus. Obviously the values for the free $\alpha$-amino nitrogen of proteins have to be treated with considerable reserve. Since the free $\alpha$-amino nitrogen is frequently assumed to indicate terminal amino nitrogen, subunits and polypeptide chains, such interpretations are also uncertain.

It is well known that glycine and terminal glycyl peptides [cf. (1)] give abnormally high values in the Van Slyke amino nitrogen apparatus. However, in the manometric Van Slyke apparatus glycine gives correct values (23). Terminal glycyl peptides have as yet not been investigated in the manometric apparatus, but their study is of considerable importance for the interpretation of the free amino nitrogen of proteins such as insulin and edestin, which are known to contain terminal glycine residues (1, 24).

*Chemical methods for the determination of amino acids.*—Modifications of Sullivan's (25, 26) colorimetric method for the determi-

nation of methionine have been reported by Csonka *et al.* (27) and by Horn *et al.* (28).

Goodwin & Morton (29) report the determination of tyrosine and tryptophane in unhydrolyzed proteins from ultraviolet absorption. The results are only of comparable accuracy because a correction has to be made for irrelevant absorption, which correction does not seem entirely justified [cf. (30)]; moreover, the findings of Crammer & Neuberger (30) on the ionization of phenolic groups in proteins have not been taken into account nor discussed. Previously, Morton (31) had attempted to determine tyrosine and tryptophane by ultraviolet absorption measurements in alkaline-stannous chloride hydrolysates of proteins.

Simpson & Traill (32) report a polarographic determination of thyroxine and of diiodotyrosine.

A colorimetric determination of canavanine has been described by Archibald (33). Canavanine gives a red color with an aquoprusside reagent, the preparation of which is described in detail. At present, the method is applicable only to the analysis of plant extracts and not to blood filtrates and urine.

Prescott & Waelsch (34) describe a micromethod for the determination of glutamic acid by converting it into its acid aldehyde by ninhydrin. The acid aldehyde is then converted into its 2, 4-dinitrophenylhydrazone which is determined colorimetrically. Aspartic acid and cystine interfere. Aspartic acid is removed by chromatographic adsorption on aluminum oxide from which glutamic acid is eluted. The interference of cystine is eliminated by calculation, i.e., applying a correction corresponding to 12 per cent of the cystine content of the protein.

Keston, Udenfriend & Cannan (35) describe a new form of isotope analysis applicable to protein hydrolysates. In this method a mixture of amino acids (hydrolysate) is treated with a reagent containing a stable or radioactive isotope to form quantitatively a stable derivative of the desired constituent. An overwhelming excess, $W$, of the unlabelled derivative (the carrier) is added and purified to constant concentration, $C_c$. If $C_r$ is the isotopic concentration of pure isotopic derivative prepared with the same reagent, the amount of derivative present is $W(C_c/C_r)$. As labelled reagent, the authors used *p*-iodophenyl sulfonyl chloride, containing radioactive iodine. The systematic application of this method to protein analysis is in progress. Preliminary results are reported for the

glycine, alanine and isoleucine content of several proteins. The values are in good agreement with other data in the literature.

Rees (8) reports modification of the Nicolet & Shinn (36) periodate oxidation method for the estimation of serine and threonine. The destruction of both serine and threonine during acid hydrolysis was studied in detail; both of these hydroxyamino acids undergo a decomposition that is directly proportional to time. The total amount of aldehyde produced on periodate oxidation was checked against ammonia simultaneously produced; the agreement in the case of the proteins investigated was good. The estimation of serine as formaldehyde in the presence of carbohydrate was studied; it seems that in the case of carbohydrate-containing proteins serine must be estimated as the difference between the periodate ammonia and threonine nitrogen. Rees' experiments indicate that histidine does not interfere with the determination of serine by the Nicolet & Shinn procedure, although Neuberger (37) has shown that histidine combines with formaldehyde.

The hydroxylysine content of various proteins was studied, since Van Slyke *et al.* (38) have shown that this hydroxyamino acid yields ammonia on periodate oxidation. Rees (8) finds that only gelatin and collagen contain hydroxylysine (1.2 and 1.1 per cent of the total nitrogen, respectively). Desnuelle & Antonin (39) likewise find hydroxylysine only in gelatin.

The determination of reactive sulfhydryl groups in the intact protein (native or denatured) is a problem fundamentally different, in significance and implications, from the determination of cysteine in acid hydrolysates of proteins. These two problems have frequently been confused, most recently by Halwer & Nutting (40). Acid-insoluble humin in hydrochloric acid hydrolysates interferes with the cysteine determination, since the humin precipitate contains appreciable amounts of cysteine, as shown first by Lugg (41). Brand & Kassell (42, 43) have found that the formation of acid-insoluble humin can be almost entirely prevented by carrying out the acid hydrolysis in the presence of urea. Under these conditions satisfactory results for both cysteine and cystine are obtained, if the photometric determination (44) is carefully followed. Halwer & Nutting (40) confirm that hydrolysis in the presence of urea prevents the formation of insoluble humin, but find that this method of hydrolysis only slightly improves the recovery of added cysteine. These low recoveries are perhaps not surprising since

Halwer & Nutting have deviated in many important details from the techniques recommended (42, 43, 44). In this case, moreover, the validity of the application of control recoveries to protein hydrolysates is questionable.

*Microbiological assays of amino acids.*—Two excellent reviews on this general subject have appeared (45, 46). For a considerable number of proteins Chibnall (1) and Brand (3) have given a systematic, detailed comparison of amino acid data, obtained by microbiological and by chemical methods. The only amino acids for which, at the present time, no microbiological methods have been developed are cysteine, cystine, alanine and hydroxyproline. Chemical methods for the determination of hydroxyproline are also unsatisfactory.

Much work has been done with various strains of the *Lactobacillus* group. There is a general tendency to further refinement of these valuable methods and greater standardization may be expected in the future. For the determination of valine, isoleucine, phenylalanine, methionine, serine and threonine the synthetic *d, l* forms are commonly used as standards. In the case of most of these amino acids it has been satisfactorily established that the growth response of the *d, l* form is exactly one half that of the natural form. An exception is the response of *Lactobacillus fermenti 36*, which can utilize *d*- and *l*-methionine equally well (47).

The first microbiological determination of tyrosine is reported by Gunness *et al.* (48) using *L. delbruckii* LD5; the results were usually 10 to 15 per cent lower than those of chemical analysis recorded in the literature. Similar results were obtained with *S. faecalis* but high concentrations of some food materials inhibited the growth of this organism.

Tryptophane has been determined with *L. arabinosus* by Greenhut *et al.* (49); the liberation of tryptophane from proteins and foodstuffs has been investigated. A protozoan has been used by Rockland & Dunn (50) for the determination of tryptophane in unhydrolyzed casein.

Threonine has been determined with *S. faecalis* by Greenhut *et al.* (51) and with *L. fermenti 36* by Dunn *et al.* (52).

The values for methionine obtained by Stokes *et al.* (53) with *S. faecalis* were rather low as compared with chemical data. A number of microbiological methods have been developed for the determination of methionine, which give results in good agreement

with those of chemical analysis. Lyman *et al.* (54) use *Leuconostoc mesenteroides* and *S. faecalis*. Riessen *et al.* (55) use *L. arabinosus* 17-5, *S. faecalis* and *L. mesenteroides* PD 60; Dunn *et al.* (47) use *L. fermenti* 36, *L. mesenteroides* P-60 and *L. arabinosus* 17-5; and Horn *et al.* (56) use *L. arabinosus* 17-5 for this determination.

The methionine content of meat was determined by Lyman *et al.* (57), the tryptophane content of blood and urine by Schweigert *et al.* (58). Hier & Bergeim (59) describe microbiological procedures for the determination of free amino acids in plasma. Values for arginine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophane, tyrosine and valine in thirty-four normal human subjects are reported; some data on the free amino acids in dog plasma and human sweat are also given (60). The methionine and isoleucine content of mammalian hemoglobins has been reported by Brand & Grantham (61).

*Ionophoresis-Chromatography.*—Martin & Synge (14), in a review on the analytical chemistry of the proteins, propose to use the term ionophoresis to describe the movement in an electric field of relatively small ions, electrophorisis for movement of large molecules and particles, and the term electrodialysis for the removal of smaller ions from large molecules and particles.

Consden, Gordon & Martin (62) have described a new method for the separation of amino acids and peptides by ionophoresis in silica jelly. They use a long thin rectangular slab of dilute, buffered silica gel which is set into a water-cooled glass trough. A carbon and a metal electrode at the ends of the slab are perfused by acid and alkaline buffer, respectively, of about the same strength as that in the gel, so that the pH of the slab remains uniform and constant. For the introduction of a protein hydrolysate, a small transverse gutter is cut out, approximately in the center of the slab. The hydrolysate, after removal of free acid, is mixed with buffered silica and set into the gutter. When a potential gradient is applied, the amino acids having a net negative charge move as a series of bands toward the anode, and those having the opposite charge toward the cathode. In order to locate the various bands, a strip of dry filter paper is laid along the length of the jelly and allowed to become saturated. After removal and drying, treatment of the paper strip with ninhydrin shows the location of amino acid bands. Such "prints," taken at suitable intervals, permit one to follow the separation. At the end of an experiment, which may last

up to a week, the jelly is cut up corresponding to the bands indicated on the prints. The concentrated filtrates from these sections are then subjected to partition chromatography on paper or other methods of amino acid analysis are used. If necessary the amino acid solutions can be completely desalted in an ingenious electrolysis apparatus designed by the authors (63); this apparatus should be generally useful.

The authors have also presented a theory of the ionophoretic separations (62). In spite of the difficulty in interpreting theoretically the effects observed, ionophoresis on silica jelly slabs should prove a very useful tool. The procedure has been applied by Synge to hydrolysates of gramicidin (64), and of "Gramicidin S" (65), and by Martin to partial wool hydrolysates (66).

Some modifications of the separation of amino acids in a conventional three-cell electrodialysis apparatus are described by Sperber (67) who adds an anion exchanger.

In the partition chromatography of acetyl amino acids on silica gel, the preparation of satisfactory samples of silica gel was shown by Gordon *et al.* (68) to be rather difficult. This question has been studied in detail by Tristram (9). He comes to the conclusion that, in order to be suitable for partition chromatography of acetyl amino acids, the water content of an iron-free gel must be increased to an optimum point at which adsorption is reduced to a minimum while the silica still retains its properties as a powder. He finds that the recovery of acetyl amino acids from gels prepared by the method of Isherwood (69) is also dependent on the water content. It, therefore, appears that there is no satisfactory criterion for the suitability of a silica gel. Each gel has to be tested by recovery experiments. Tristram's standard procedure is to subject a comprehensive mixture of acetyl amino acids to chromatographic analysis and to reject any gel which gives a recovery of less than 95 per cent for any constituent.

According to Consden *et al.* (70), nonadsorptive silica gels such as are useful for partition chromatography of acetyl amino acids (9, 68), do not permit useful separations of DNP-amino acids (DNP=2,4-dinitrophenyl) by the solvent systems of Sanger (24). When such gels are used in chromatograms, the DNP-amino acids all run together as fast bands (70). Nevertheless, the samples of silica gel used by Sanger (24, 71) gave the expected separations of the DNP-amino acids (70). According to Consden *et al.* (70), the

action of Sanger's gels must be attributed to the super-imposition of rather strong adsorption effects upon the effects due to distribution between the two liquid phases.

Many technical details relating to the partition chromatography of acetyl amino acids may be found in the excellent paper by Tristram (9). In view of the anomalous behavior of methionine, he restricts the method to the determination of phenylalanine, the leucines, valine, proline, tyrosine, and alanine. The values obtained for insulin, edestin, casein and β-lactoglobulin have been submitted by Tristram to control by the analysis of corresponding artificial mixtures of amino acids. The probable accuracy of the method is discussed with special reference to insulin and edestin. Tristram (9) regards the values reported for zein, gliadin, ovalbumin, gelatin and collagen as provisional, because they have not been submitted to control analysis. In many instances, the results are in good agreement with values obtained by microbiological methods.

Hamoir (72) suggests the use of a flocculum of silver sulfide as adsorbent for the chromatography of amino acids in aqueous solution. His investigations indicate new preparatory and analytical possibilities and methods for eliminating the adsorption of organic compounds at the surface of the mineral precipitates.

Elsden & Synge (73) have suggested the use of potato starch as a supporting medium for the aqueous phase in the partition chromatography of free amino acids and peptides. Sufficient material may be fractionated on starch columns to permit further investigation of the components of a mixture by conventional microchemical techniques. Synge (74) used a starch column for the isolation of *l*-valylglycine from a partial hydrolysate of gramicidin. Glycine, alanine, valine, leucine and tryptophane ran as bands on starch chromatograms, but quantitative experiments on recovery of amino acids from such columns were not carried out. Moore & Stein (75) have recently extended the studies on starch columns and have established a procedure for their employment on a quantitative basis. The effluent from the column has been collected in small aliquots on an automatic fraction cutter and each aliquot analyzed by a colorimetric ninhydrin method (76) developed for this purpose. The results have been expressed in terms of effluent concentration curves. Where separation of the components is complete, a discrete, fairly symmetrical curve of the Gaussian type is obtained for each individual amino acid. The amino acids are thus

spread out to yield a "spectrum" of curves. The high resolving power of columns of this type has been demonstrated by the complete separation of leucine from isoleucine. The technique appears to be capable of yielding quantitative and qualitative data on the composition of mixtures of amino acids and related compounds. Using 1:1 *n*-butanol-benzyl alcohol as the organic solvent, values have been obtained for phenylalanine [3.78 per cent], leucine [15.5 per cent], and isoleucine [5.86 per cent (76)] in hydrolysates of $\beta$-lactoglobulin. Preliminary experiments have shown that the method can be extended to cover the whole amino acid "spectrum." With the solvent systems studied in the first experiments the resolution of the slower moving components has not been complete in all instances. The techniques developed by Moore & Stein show great promise of becoming a standard procedure for the accurate determination of a considerable number of amino acids in protein hydrolysates and for the isolation and separation of peptides from partial hydrolysates. Their method may well supersede the partition chromatography of acetylated amino acids on silica gel. The automatic fraction-cutter designed by these authors should prove generally useful for all kinds of chromatographic work.

Claesson (77) has greatly increased the power of the adsorption analysis technique developed by Tiselius, employing continuous recording of the concentration of the solutes emerging from the adsorption column. The refractive index of the emerging solution is determined by an interference refractometer. As yet, only relatively simple amino acid mixtures have been analyzed by these methods, employing "frontal analysis" or "displacement development." The method now, however, shows great promise for the study of more complex mixtures. A general review of this type of chromatographic analysis is given by Tiselius (78).

The successful separation of amino acids on paper chromatograms by Consden, Gordon & Martin (79) led to attempts to employ similar methods quantitatively. Martin & Mittelmann (80) have put a number of one dimensional chromatograms on a sheet of paper and developed the end ones with ninhydrin. The untreated parts were cut at the proper places and extracted with copper-phosphate-borate buffer. The soluble copper complexes remained in the supernatant and the amino acids were determined by polarographic methods. This procedure has been applied so far to simple

amino acid mixtures such as obtained from hydrolysis of "Gramicidin S." Consden, Gordon & Martin (81) have used paper chromatograms for the identification of small peptides in complex mixtures. The method involves the identification of the small peptides on paper chromatograms, cutting out, washing out, and hydrolysis before and after deamination. Paper chromatograms have also been used for the identification of amino acids derived from cystine in chemically modified wool (82). Dent (83) has applied paper chromatography to the investigation of amino acids and peptides in normal and pathological urines.

*Peptides.*—Prelog & Wieland (84) have described the synthesis of a peptide, *l*-lysyl-glycyl-glycyl-*l*-glutamic acid. Plentl & Page (85) report the synthesis and enzymatic degradation of *l*-tyrosyl-*l*-lysyl-*l*-glutamyl-*l*-tyrosine. Both peptides should be of interest to the physical chemist. A peptide of *p*-aminobenzoic acid (PAB) has been isolated from yeast by Ratner *et al.* (86); it consists of one terminal PAB residue, one amide group, ten or eleven *l*-glutamic acid residues, and one unknown amino acid residue, presumably acidic in nature. This peptide of Ratner *et al.* may be related to the so-called vitamin $B_c$ conjugate (folic acid conjugate) which has been shown by Pfiffner *et al.* (87) to be pteroylhexaglutamyl glutamic acid.

The sequence of the amino acid residues in "Gramicidin S" has been reported by Consden *et al.* (70, 88); Synge (65) previously established the constituent amino acids as *l*-leucine, *l*-ornithine, *d*-phenylalanine, *l*-proline and *l*-valine, and found that there were no $\alpha$-amino or carboxyl groups present. That the $\delta$-amino group of the ornithine residue is free was shown by Sanger (71) with his dinitrofluorobenzene method (24). Partial hydrolysates of "Gramicidin S" were fractionated on two-dimensional paper chromatograms (79), and by ionophoresis (62). From the isolation, identification and synthesis of four dipeptides and three tripeptides, (81, 89), the sequence of the amino acid residues in the cyclopeptide was established as follows (70, 88):

-$\alpha$-*l*-valyl-*l*-ornithyl-*l*-leucyl-*d*-phenylalanyl-*l*-prolyl-

X-ray data obtained by Crowfoot & Schmidt, together with the above findings, indicate that "Gramicidin S" is either a simple cyclopentapeptide or a cyclodecapeptide (70, 88).

Martin (66) has fractionated a wool hydrolysate by ionophoresis on silica gel (62). Approximately twenty-five dipeptides involving aspartic and glutamic acids were identified. Among these dipeptides were aspartyl-aspartic acid and glutamyl-glutamic acid. Martin concludes that the structure of wool is more complicated than has been suggested by Astbury and does not conform to the Bergmann-Niemann hypothesis. According to Martin (66), Astbury has stated that he looks upon the structure of proteins "constructively and with the eye of stoichiometric faith"; Martin, apparently lacking this eye and approaching this problem analytically, is unable to find the same degree of order and simplicity postulated by Astbury (66).

The phosphopeptone of casein has been reinvestigated by Nicolet & Shinn (90). It appears to be an octapeptide, which contains, besides one unidentified unit, two units of glutamic acid, two of isoleucine, two of phosphoserine and one of serine. Partial hydrolysis yielded phosphoseryl-glutamic acid, the structure of which was proven by phosphatase and periodate reactions.

*Blood group substances.*—Recent developments in amino acid analysis (microbioassays, paper chromatography) have been applied in elucidation of the structure of the blood group "A-substance." The blood group "A-substance" from hog stomach is a mucoprotein [for definition cf. (91)], i.e., a carbohydrate-amino acid complex, about one-fourth of which is composed of amino acids (92). A noteworthy advance in the study of this complex was made by Kabat & Bezer (93) who found that the "A-substance" could be specifically precipitated by its corresponding isoantibody in the serum of normal or immunized individuals. It was found (94, 95) that specimens of the highest purity and activity could be obtained only when the stomachs of individual hogs were used. By the phenol method of Morgan & King (96), from each of ten hog stomach linings, highly purified products were obtained in the same yield, which were identical with respect to nitrogen, reducing sugar, glucosamine, and acetyl content and had the same relative viscosity; yet only seven exhibited blood group A activity, whereas the remaining three were devoid of A (as well as of B) activity. Hitherto, gastric mucin obtained from random pools of hog stomachs has been extensively used as a source of "A-substance," and the purified products obtained have been of lower activity, since they were composed, in part, of an inactive material (95). Since Morgan & Waddell (97) had noted that "A substance" from pools

of hog stomachs showed both A and O activity, the isolation of an A-inactive product from certain individual hog stomachs (95) suggested that the inactive material might be blood group O substance.

Indeed Aminoff, Morgan & Watkins (98) could show that the partially purified mucoproteins isolated from individual hog stomachs were serologically different. From twenty-four hog stomachs they obtained fourteen preparations which possessed A specificity only, whereas the remaining ten preparations showed only specific character O. None of these preparations showed both A and O specificity and no specimen was without either A or O character (98). Very recent studies indicate that material from certain individual hog stomachs possesses both A and O specificity [Bendich, A., Kabat, E. A., and Bezer, A. E. (In press)].

Additional evidence that the highly purified, but "A-inactive" material isolated from certain hog stomachs (95) is blood group O substance was obtained by Kabat *et al.* (99), who found that their purified inactive preparations reacted as well or better than their active "A-substance" with type XIV antipneumococcal horse serum which is known to agglutinate cells of blood groups A, B and O.

None of the highly purified and serologically uniform preparations of the blood group substances have as yet been analyzed for their amino acid content. However, Morgan (100) established qualitatively by means of paper chromatography the presence of at least fifteen amino acids in an acid hydrolysate of "A-substance." It seems probable that threonine and hydroxyproline were present in higher concentrations than are usually found in protein hydrolysates. Cystine appears to be absent. An actual isolation of threonine from "A-substance" has been reported by Freudenberg *et al.* (101). Brand & Saidel (102) studied the amino acid composition of a purified preparation of "A-substance." Twelve amino acids determined by microbiological methods accounted for approximately half of the protein nitrogen. Proline, glycine and serine were present in appreciable amount, but neither threonine nor hydroxyproline was determined. Detailed data on the amino acid composition of highly purified and serologically uniform preparations of blood group substances A and O will be awaited with great interest, particularly since these substances seem to be high in hydroxyamino acids.

On the basis of enzymatic experiments, Meyer (91) has con-

cluded that blood group A activity is entirely dependent on the polysaccharide moiety regardless of the peptide groups attached to it. On the other hand, Morgan (103) has recently reported on the chemical changes brought about by the action of mixed enzyme preparations obtained from *Cl. welchii* on "A-substance" from commercial gastric mucin which is composed of A and O substances. He finds that the enzymatic inactivation of the "A-substance" is accompanied, among other changes, by an increase in free amino nitrogen which, to considerable extent, is due to the liberation of free $\alpha$-amino acids. It therefore seems possible that the protein moiety of blood group A and O substances is concerned, either alone or together with the carbohydrate moiety, in the activity and specificity of these substances. Further developments in this important field will be awaited with keen interest.

*Miscellaneous.*—Blass & Macheboeuf (104) report two new amino acids in the cholera vibrio: aminoadipic acid and hydroxyaminoadipic acid. New syntheses of *d,l*-tryptophane (105), of 5-methyl-tryptophane (106), of lysine anhydride (107), of *d,l*-methionine (108), of *d,l*-serine and its methylester (109) and of *l*(+)-glutamine (110) have been described. Dakin (111) reports the synthesis of $\delta$-chloroleucine which is converted by treatment with alkali into $\gamma$-methyl proline. Fox (112) describes derivatives of 3,5-diiodotyrosine, Reineke & Turner (113) the formation of thyroxine from diiodotyrosine, Sealock (114) the preparation of *d*-tyrosine and of *d*-3,4-dihydroxyphenylalanine. A synthesis of phosphoglycocyamine, similar to that of phosphocreatine, is reported (115). The stereoisomeric salts of leucine methylester have been investigated by Weil & Kuhn (116). Anslow, Simmonds & du Vigneaud (117) report the synthesis of the 4 stereoisomers of cystathionine [S-($\beta$-amino-$\beta$-carboxyethyl)-homocysteine], a thio ether, which is an intermediate in the conversion of methionine to cystine (118, 119, 120). The thio ether which is related only to the natural amino acids (*i.e.*, contains the *l*-cysteine or *l*-homocysteine moiety, respectively) is designated as *l* (+)-cystathionine, and its optical isomer as *d* (−)-cystathionine (related to *d*-cysteine and *d*-homocysteine). For the remaining two isomers, which are diastereoisomers of the first pair, the name allocystathionine is suggested: *l*(−)-allocystathionine for the ether related to *l*-cysteine and *d*-homocysteine; *d* (+)-allocystathionine for the ether related to *d*-cysteine and *l*-homocysteine. An excellent review on glutamine has been contributed by Archibald (19).

## Proteins

*Modification of proteins by chemical reagents.*—An extensive review of this field has been given by Herriott (121); and the reactions of amino acids and proteins with formaldehyde have been reviewed by French & Edsall (122). Since these reviews were published, Fraenkel-Conrat & Olcott (123) have reported additional evidence for the participation of guanidine groups in reaction with formaldehyde at 70°, in neutral or acid solution. Protamine sulfate, as well as simple substituted guanidine salts, bind up to two moles for each guanidine group; unsubstituted guanidine salts can bind as much as three moles of formaldehyde. If the reaction is carried out at moderately high protamine concentrations, there is evidence from osmotic pressure measurements of increase in average molecular weight of the protamine; this would appear to indicate intermolecular condensation through cross linking methylene groups. Steinhardt, Fugitt & Harris (124) studied the effect of formaldehyde on the titration curve of wool keratin, and interpreted the results in terms of reaction with amino and guanidyl groups of the protein.

Reitz *et al.* (125) introduced sulfate groups into proteins, both by the direct action of cold concentrated sulfuric acid, and by the action of pyridine chlorosulfonic acid (126). The former yielded acid sulfates of the hydroxyl groups of serine, threonine and hydroxyproline. The sulfhydryl group of cysteine reacted to form the thiosulfate. In the presence of pyridine chlorosulfonic acid, under analogous conditions at 70 to 80° for two and one half hours, all these and other groups reacted, including the phenolic hydroxyl, primary amide, amino, guanidyl and indole groups, which were transformed to sulfates or sulfamates. The stability of the various links was investigated.

Fraenkel-Conrat & Olcott (127) methylated the carboxyl groups of several proteins almost completely by treatment with methanol and mineral acid (0.02 to 0.1 *N*) at room temperature for one to six days. This relatively mild treatment apparently did not affect amino, phenolic, thiol, or indole groups. Peptide and amide bonds were also unaffected. Esterification with higher alcohols was also obtained under similar conditions, but was slower and less complete than with methanol.

Extensive reports on the action on proteins of the nitrogen and sulfur mustard gases and oils ($\beta$-chloroethyl amines and sulfides), obtained in secret research during the war, are now beginning to

be released. General summaries of results obtained have been given by Gilman & Philips (128), and by Dixon & Needham (129).[1] These compounds owe their activity to a fundamental chemical reaction, namely intramolecular cyclization to form a cyclic onium cation, as follows:

$$RZ\text{—}CH_2\cdot CH_2\cdot Cl \rightarrow \overbrace{RZ^{+}\text{—}CH_2\cdot CH_2}^{} + Cl^{-}$$

where Z represents either sulfur or nitrogen. This cation reacts readily with anions and various uncharged molecules. Rapid reaction takes place with water, but if other substances are present, they can react competitively; many organic groups react much faster than water. Amino, imidazole, sulfhydryl, sulfide, phenolic, and imino groups of amino acids, peptides and proteins react, giving alkyl derivatives. Herriott, Anson & Northrop (130) have studied the rate of reaction of mustard gas (H) with thirteen proteins. All were found to react, although the most reactive combined with H about one hundred times as fast as the least reactive. The carboxyl groups of all proteins studied reacted with H at pH 6. In most cases, the number of carboxyl groups covered was found to be approximately equal to the number of H residues bound. In general these authors did not find the amino groups of proteins to react, with the possible exception of those in yeast hexokinase. It should be added that there are some discrepancies between the findings of workers in different laboratories, the significance of which cannot be fully evaluated until further reports are published.

Mayer & Heidelberger (131) have phosphorylated crystallized horse serum albumin with phosphorus oxychloride in the cold. As many as sixty-one phosphoryl groups were introduced per albumin molecule, when the reaction was carried out at pH 8.5 to 9, alkali being added during the process to maintain constant pH. Only about thirty groups were introduced if the reaction was carried out in borate buffer. Many of the groups introduced were subsequently split off spontaneously, even in the cold, but some remained firmly bound. The isoelectric point of the phosphorylated protein was lower than that of the original albumin, and many preparations were insoluble near the isoelectric point. The sero-

[1] Dixon & Needham give a general discussion of other vesicants also.

logical specificity was also modified. The chief sites of phosphorylation in the protein appear to be amino and phenolic hydroxyl groups.

## Blood Plasma Proteins

*Methods of plasma protein fractionation.*—Extensive developments in the large scale fractionation of blood plasma proteins have occurred during the War. The first detailed report, on the chemical methods involved, has now been published by Cohn *et al.* (132). The process involves precipitation with ethanol at low temperatures (0° to $-5°$ C.) under carefully controlled conditions of pH, ionic strength, ethanol concentration, protein concentration, and temperature. These five variables are all subject to independent variation, within the limits imposed by the necessity for preserving the proteins in undenatured form, so that a very wide variety of possible conditions for separation is obtainable. The ionic strength ($\Gamma/2$) is never higher than 0.16 moles per liter in the process as at present developed and at some steps it is as low as .001. There are several advantages of these processes over the usual salting out techniques,[2] namely: (*a*) the effects of salts on different proteins at low ionic strengths are, in general, greater and more specific than at the high ionic strengths employed in salting out; (*b*) the ethanol used as precipitant lowers the dielectric constant of the solution and thereby enhances the interactions between salt ions and proteins, which lead to changes in solubility; under these conditions, even the albumin components of plasma behave like globulins, being insoluble in the absence of salt, when near their isoelectric points, owing to the presence of ethanol in the solvent; (*c*) ethanol can be readily removed by freezing the resulting preparations and drying them from the frozen state, a procedure which is much more adaptable to large scale fractionation than dialysis, which is necessary to remove the high concentrations of salt obtained by the salting out methods. Some proteins, notably the lipoproteins of plasma, are denatured on freezing and drying from the frozen state, but nearly all other fractions can be obtained, apparently in undenatured form, by these procedures.[3]

[2] Reviews of recent developments in plasma fractionation are given by Cohn (133, 134, 135), Edsall (136), and Mulford (137).

[3] In connection with the effects of alcohol on proteins, it is interesting to note that pepsin is soluble, in acid solution, even in 65 per cent alcohol (138). It may be readily crystallized from 20 per cent alcohol at room temperature. The crystals ap-

The fractionation of human plasma has yielded five principal fractions in the latest methods employed: these are denoted as Fractions I, II+III, IV-1, IV-4, and V. The conditions of separation, and the electrophoretic analysis, of these fractions are given in Table I. Some comment is necessary on certain items in this Table. Fraction I contains not only fibrinogen, but also a globulin

TABLE I

SEPARATION AND ELECTROPHORETIC COMPOSITION OF PRINCIPAL FRACTIONS OF HUMAN PLASMA BY METHOD 6

| Fraction | Conditions of Separation | | | | | Electrophoretic Composition gm. per liter of plasma (estimated in plasma) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | pH | Γ/2 | Temp. °C. | Mole fraction Ethanol | Protein in system, gm./l. | Albumin | α | β | γ | Fibrinogen | Total |
| Plasma | 7.4 | .16 | — | — | 60.3 | 36.3 | 9.2 | 10.6 | 7.2 | 2.5 | 65.8 |
| I | 7.2 | .14 | −3 | .027 | 51.1 | 0.2 | 0.3 | 0.5 | 0.3 | 2.1 | 3.4 |
| II+III | 6.8 | .09 | −5 | .091 | 30.0 | 0.8 | 1.1 | 9.1 | 7.0 | 1.0 | 19.0 |
| IV-1 | 5.2 | .09 | −5 | .062 | 15.8 | 0 | 4.5 | 0.5 | 0.1 | 0 | 5.1 |
| IV-4 | 5.8 | .09 | −5 | .163 | 10.1 | 0.9 | 2.7 | 2.2 | 0 | 0 | 5.8 |
| V | 4.8 | .11 | −5 | .163 | 7.5 | 29.9 | 1.3 | 0.3 | 0 | 0 | 31.5 |
| VI | 4.8 | .11 | −5 | .163 | 0.2 | 0.8 | 0.2 | <.1 | 0 | 0 | 1.0 |
| Total | — | — | — | — | — | 32.6 | 10.1 | 12.6 | 7.4 | 3.1 | 65.8 |

Data from Cohn, Strong, Hughes, Mulford, Ashworth, Melin & Taylor (132); see especially Tables II and IX of their paper. Electrophoretic figures in the top row are for measurements directly on plasma; those in the bottom row are summations from the distribution in the plasma fractions, multiplying by the appropriate factor for the percentage of total plasma protein represented in the fraction.

Electrophoretic components of plasma with mobilities close to that of fibrinogen are resolved with difficulty. The value of 2.5 gm./l., for fibrinogen in whole plasma, is derived from Edsall, Ferry & Armstrong (175), who base their estimates on the amount of protein clottable with thrombin [see also Morrison (174a)]. The remainder of the protein with the same mobility is included with the β-globulins. A similar situation occurs with Fraction II+III.

Satisfactory resolution of the $\alpha_1$-globulin from albumin in Fraction IV-1 has not been accomplished, and since probably less than 25 per cent of the fraction is albumin, any components with mobilities similar to that of albumin have been included with the α-globulins. Further studies on this point are in progress.

which accelerates the clotting of the blood of patients with hemophilia, both *in vitro* and *in vivo*, and a non-clottable portion which

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pear as needles or plates which can be transformed into the usual hexagonal bipyramids by recrystallization from water. The enzyme is quite stable in 20 per cent alcohol at pH 2 and 25° C., but is slowly inactivated by high concentrations of alcohol. It is stable for several hours in 65 per cent alcohol at pH 4 to 5, but is rapidly inactivated in more acid solution.

is distinguished by its very great change of solubility with temperature (139). The nature of the antihemophilic factor is still obscure; experimentally it has been very difficult to separate from fibrinogen, although it is relatively stable to heat at temperatures in the range 55° to 60° C. at which fibrinogen is rapidly coagulated. A possible clue to the nature of this fraction is provided by the recent work of Chargaff & West (140), who have separated a very small amount of a high molecular weight fraction from normal plasma by high speed centrifugation (at 31,000 *g*). The resulting fraction significantly accelerates the clotting time of hemophilic blood or plasma. The corresponding fraction from hemophilic blood was minute in amount compared to that from normal blood. The *in vitro* assay of the antihemophilic factor in Fraction I and other plasma fractions has been described by Taylor, Davidson, *et al.* (141), and the results of some successful clinical injections of this fraction are described by Minot, Davidson *et al.* (142). Similar clinical results, in a large number of patients, have been obtained by Diamond, in work to be published shortly.

Fraction II+III contains a wide variety of important components, chiefly $\beta$- and $\gamma$-globulins, and includes prothrombin, the isoagglutinins, plasminogen (fibrinolysin precursor), nearly all the antibodies of plasma, the component $C_1'$ of complement, and a lipoprotein of plasma (X-protein) which is electrophoretically a $\beta_1$ globulin. Fraction IV-1 contains another lipoprotein which is, however, an $\alpha$-globulin, as well as several other components. Fraction IV-4 consists chiefly of $\alpha$- and $\beta$-globulins, with some albumin. It contains serum esterase, and a globulin component which shows a specific capacity to combine with inorganic iron (135, 143).[4] Most of these globulins are virtually lipid-free. Fraction V, which is not far from being pure albumin, when first precipitated, is easily reprecipitated to give a product containing 97 to 99 per cent albumin by electrophoresis; and it includes 75 to 80 per cent of all the albumin in human plasma. Albumin was

[4] The work of Granick (144) has shown that the feeding of iron to guinea pigs leads to the formation of apoferritin in the intestinal mucosa, the iron being absorbed as ferritin in the mucosa. Further absorption of iron from these ferritin deposits into the body tissues occurs slowly, over a period of days. The iron-binding globulin of Fraction IV-4 may serve as the intermediary transporting agent between the mucosal ferritin and the body tissues. The present state of our knowledge of ferritin has been reviewed by Granick (145) and by Michaelis (146).

prepared by these methods in large quantities, during the War, by several pharmaceutical houses for use in the Navy and Army.[5]

The crystallization of both human and bovine serum albumin from ethanol-water at low temperatures has been achieved by Cohn & Hughes (unpublished).

Several of the fractions contain so many distinctly different components that further subfractionation is necessary. The subfractionation of Fraction II+III, in particular, has been studied by Oncley *et al.* (147, 148). Some of the general principles, on which the present separation procedure is based, are as follows:

(*a*) The $\beta_1$-lipoprotein of plasma [X-protein of Pedersen (149)], is soluble at pH $7.4 \pm 0.2$, even at relatively high ethanol concentrations (17 to 20 per cent), while nearly all other components of this fraction are insoluble under these conditions, at low ionic strength.

(*b*) Prothrombin and plasminogen (the precursor of the proteolytic enzyme of plasma) are insoluble at pH 5.2-5.4, even in the absence of alcohol and at ionic strengths between 0.05 and 0.10. This Fraction (III-2,3) separated under these conditions also contains component $C_1'$ of complement (150) which is, however, very unstable under the conditions of separation.

(*c*) The isoagglutinins are also insoluble at pH 5.2 to 5.4, at very low ionic strengths, but are more readily dissolved by a slight increase of ionic strength than the components of Fraction III-2,3. The isoagglutinin fraction, which must, of course, be obtained from suitably typed blood, is denoted as III-1. Fraction III-1 contains not only anti-A and anti-B, but also anti-Rh isoagglutinins, provided the latter are present in the starting material. The concentration of activity achieved by fractionation has permitted the preparation of anti-Rh typing materials of satisfactory potency from plasmas which could not have been used as such for typing purposes. The preparation of anti-B isoagglutinins from pooled type A plasmas is a straightforward procedure. The relatively small number of type B donors in the population, however, was insufficient to provide a sufficient amount of anti-A isoagglutinin. This problem was solved by Melin (151) who introduced the practice of mixing pooled O and B bloods, the B cells thereby absorbing anti-B isoagglutinins from the O plasma, leaving the anti-A isoagglutinins in good titer and avidity. The

[5] The history of the plasma fractionation program during the war has been told by Cohn and associates (135).

resulting plasma could then be fractionated to yield a concentrated and very active anti-A preparation. Type A blood must be rigorously excluded from the O+B pool, if satisfactory activity is to be attained.

It should be noted that only about one per cent of the protein of even the most purified samples of Fraction III-1 is actually isoagglutinin.[6] However, for practical purposes these preparations have yielded highly active blood typing reagents.

(*d*) Most of the $\gamma$-globulins are least soluble at pH values near 7. At pH near 5.2, they are very soluble, at low ionic strength. One portion of the $\gamma$-globulins, however, is precipitated in this pH region by very moderate increase of ionic strength at an ethanol concentration of 17 per cent and at $-5°$ to $-6°$ C. (153). In the latest method of fractionation (147) of Fraction II+III, this portion of the $\gamma$-globulin is denoted as II-3, whereas the other, and larger, portion of the $\gamma$-globulin is precipitated at pH near 7 and is denoted as II-1,2. Both these fractions contain a high concentration of a large number of different antibodies, including antibodies to diphtheria, influenza, mumps, whooping cough, scarlet fever, poliomyelitis, lymphocytic choriomeningitis, certain streptococci, vaccinia and typhoid (135, 154). Clinically, this fraction has found extensive use for passive temporary immunization against measles (155, 156) and against infectious hepatitis (157, 158, 159).

The enzymatic digestion of human $\gamma$-globulin has been studied with pepsin by Bridgman (160) and with bromelin and papain by Petermann (161). Pepsin digestion leads predominantly to breakdown into molecules of half the original size. Practically all the antibody activity is retained, although the typhoid "O" agglutinin is destroyed by the digestion. Prolonged digestion leads to the production of still smaller molecules, and eventually to dialyzable breakdown products. Digestion by papain or bromelin leads to the formation of quarter molecules ($s_{20} = 4.1$ S) with only a small increase in nonprotein nitrogen. Electrophoretically, the digestion products are heterogeneous, giving a series of different components, in sharp contrast to the pepsin digestion products which are homogeneous by electrophoresis and practically identical in mobility with the undigested globulin. The quarter molecules

[6] Personal communication from E. A. Kabat; determinations were made by the procedures of quantitative immunochemistry (152).

still possess some antibody activity, although considerably less, with respect to most antibodies tested, than the original globulin. Deutsch, Petermann & Williams (162) developed a combined pepsin digestion and fractionation procedure to recover half size $\gamma$-globulin antibodies from an initial fraction containing $\beta$-and $\gamma$-globulins.[7] The half-size antibodies give less viscous solutions and diffuse more rapidly than the original molecules, and these modified properties may be valuable for clinical use.

Separation of a globulin with electrophoretic mobility slightly greater than that of ordinary $\gamma$-globulin has been reported by Deutsch, Alberty & Gosting (163). They denote this new fraction as $\gamma_1$-globulin (other authors have denoted it as $\beta_2$) and report, in detail, methods for its separation from the bulk of the $\gamma$-globulin fraction (which they denote as $\gamma_2$-globulin).

The ammonium sulfate fractionation of bovine colostrum and plasma has been studied by Smith (164). Immune lactoglobulin is the predominant protein in bovine colostrum, and was isolated in electrophoretically homogeneous form. Its molecular weight is high (in the range of 160,000 to 190,000) and it differs greatly from $\beta$-lactoglobulin of milk. The immune activity of bovine plasma is present in both T- and $\gamma$- components. Both these fractions were isolated and characterized by elementary composition, isoelectric points and diffusion constants.[8]

*Size and shape of plasma protein molecules.*—A systematic study of blood plasma fractionation has revealed a very much larger number of individual components than were recognized even a few years ago. Human plasma contains at least four different kinds of $\beta_1$-globulin, and several different kinds of $\alpha$-globulin. A very large number of different $\gamma$-globulins must be present, corresponding to the great range of different antibody activities found in the $\gamma$-globulin fraction (154). Moreover, the purified $\gamma$-globulin fraction, although electrophoretically homogeneous, is far from homogeneous in the ultracentrifuge. About 85 per cent of

[7] This fraction was denoted as III-1 in an earlier fractionation method, 3C (147, 148). The $\gamma$-globulin present in this fraction was later separated in Fraction II-3 in method 9.

[8] Smith (165) has obtained two distinct "immune globulins" from bovine whey. They were isolated in electrophoretically homogeneous form; one is a euglobulin, the other a pseudoglobulin. They differ also in amino acid composition and mobility.

the material is of sedimentation constant ($s_{20,w}$) = 7 S; the remainder sediments more rapidly (about 10 S). Comparison of the sedimentation and electrophoretic diagrams of other plasma fractions reveals a complex variety of components. There is no simple correlation between the components revealed by the two methods of analysis (135, 166).

TABLE II

PROTEIN COMPONENTS OF NORMAL HUMAN PLASMA CHARACTERIZED BY PHYSICAL CHEMICAL METHODS*

| Electrophoretic component | Fraction | Approximate amount in plasma (gm./l.) | Sedimentation constant $s_{20,w}$ | Specific volume $V$ | Intrinsic viscosity $H_s \times 10^2$ | Frictional ratio $f/f_0$ | Molecular weight $M$ | Approximate dimensions (Ångstroms) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Length | Diameter |
| Albumin | V | 32 | 4.6 | 0.733 | 4.2 | 1.28 | 69,000 | 150 | 38 |
| $\alpha_1$-globulin | IV-1 | 2† | 5.0 | .841 | 6.6 | 1.38 | 200,000 | 300 | 50 |
| $\alpha_1$-globulin | IV-6 | 1 | 9. | .693 | 9.2 | 1.58 | (300,000) | — | — |
| $\beta_1$-globulin | IV-7 | 2 | 5.5 | .725 | 5.5 | 1.37 | 90,000 | 190 | 37 |
| $\beta_1$-globulin | III-0, III-2 | 2 | 7. | .74 | — | — | (150,000) | — | — |
| $\beta_1$-globulin | III-0 | 1 | 20. | .74 | — | — | $5 \times 10^5$ to $10 \times 10^5$ | | |
| $\beta_1$-globulin | III-0 | 2† | 2.9‡ | .950 | 4.1 | 1.7‡ | 1,300,000 | 185 | 185 |
| $\beta_1$-globulin | III-1 | 2 | 7. | — | — | — | (150,000) | — | — |
| $\gamma$-globulin | II | 5 | 7.2 | .739 | 6. | 1.38 | 156,000 | 235 | 44 |
| $\gamma$-globulin | II | 1 | 10. | .739 | — | — | (300,000) | — | — |
| Fibrinogen§ | I-2 | 2 | 9. | — | 25. | 1.98 | 400,000 | 700 | 38 |

* Oncley, Scatchard & Brown (166).

† These two globulins are lipoproteins containing 35 per cent lipid for the $\alpha_1$-globulin and 75 per cent lipid for the $\beta_1$-globulin. The other components contain little or no lipid.

‡ This is the sedimentation constant obtained in 0.5 $M$ sodium chloride solution, when corrected in the usual manner. The $f/f_0$ value given here is the value for sedimentation, correcting for the partial specific volume of the hydrated protein (0.97), using the equation of Kraemer [equation 124, p. 65, (252)]. A solvation of 0.6 grams of water per gram of protein was used for this calculation, and the molecule was assumed spherical.

§ Holmberg (201) estimates a molecular weight, for human fibrinogen prepared by salting out, of 700,000 from sedimentation and diffusion. From double refraction of flow and other data, J. T. Edsall, J. F. Foster & H. Scheinberg (unpublished) estimate a molecular weight near 500,000 for human fibrinogen.

The properties of the principal components of certain fractions, which have been well characterized by physicochemical methods, are listed in Table II. The methods employed include ultracentrifugal analysis, viscosity and diffusion measurements, and, in certain cases, double refraction of flow. It is notable that all of the principal plasma protein components have very nearly the same molecular cross sectional diameter, approximately 35 Å. The length of the molecule, however, varies enormously from one individual

protein to another, from about 700 Å in fibrinogen to approximately 150 Å in albumin. In a class by itself is the $\beta_1$-lipoprotein, first recognized by McFarlane (167), and denoted by him as the X-protein of plasma. Later it was intensively studied by Pedersen (149), who found that its sedimentation rate varied enormously with the density of the solvent, falling to zero in a solvent of density 1.04, this value thus being equal to the density of the protein. It was apparent that this component must contain a very large amount of lipid; the recent analyses of Oncley, Melin *et al.* (147), indicate that it contains approximately three times as much lipid as protein. The lipid portion includes cholesterol, phosphatides, some fatty acids (147), and most of the estrogens of plasma (168).

The molecule appears to be rather heavily hydrated, and to be approximately spherical. In spite of its very high lipid content, it dissolves readily in dilute salt solution to a concentration of 10 per cent or more. The $\alpha_1$-lipoprotein of Fraction IV-1 also contains considerable amounts of cholesterol, but its total lipid content is only about 35 per cent.

Pedersen's comprehensive ultracentrifugal studies (149) are also notable for the information they supply concerning fetuin, already briefly discussed in last year's review (169). This globulin, with a molecular weight near 50,000 and a high dissymmetry ratio ($f/f_0$ = 1.6–1.8), is a predominant component in the serum of bovine fetus, and is present in considerable amounts in the sera of young calves, of foals, and of sheep's fetus. Very little is present in human umbilical cord serum or in rabbit's fetus. Pedersen (149) also reported an extended series of studies on the ammonium sulfate fractionation of the sera of man, sheep, rabbits, cows, and horses, systematically varying pH, ionic strength, and other conditions.

*Amino acid composition of plasma proteins.*-Comprehensive data are now available on the amino acid composition of some of the purified plasma protein fractions. Shemin (170) has determined values for aspartic and glutamic acid, tyrosine, leucine, and glycine in purified human and bovine serum albumin. The isotope dilution procedure is at present to be regarded as the most satisfactory yardstick for checking the validity of other procedures by comparative determinations on given amino acids in the same protein preparations. It is therefore encouraging to find, for these and

other proteins that have been investigated, that very satisfactory correspondence is obtained between results derived by the isotope dilution and microbiological assay techniques. Brand (3, 13) has made extensive and nearly complete amino acid analyses of several protein fractions, utilizing chiefly microbiological and colorimetric methods, and the results for several human plasma proteins are given in Table III. It should be noted that while the albumin and $\gamma$-globulin fractions they studied were highly pure, the fibrinogen preparation was only 87 per cent clottable with thrombin, and the $\alpha$- and $\beta$-globulin preparations were by no means pure. Furthermore, each of these latter fractions represents only one of the several $\alpha$- and $\beta$-globulins now known to be present in human plasma. A systematic comparison of the composition of human and bovine serum albumin has been made by Brand (3). The molecular weights and other physical properties of the two proteins are very nearly identical and their content of many amino acids is also identical. However, there are distinct differences between them in the content of methionine, tryptophane, serine, threonine, valine, leucine, isoleucine, and phenylalanine.

Smith, Greene & Bartner (171) have carried out amino acid and carbohydrate analyses on several immune proteins from the cow, man and horse. All these proteins show a fundamentally similar composition. They contain carbohydrate with an approximate ratio of 2 hexose residues to 1 of hexosamine. Tryptophane, leucine, valine, and phenylalanine content are very similar for all these fractions, but there are differences, some of which seem to be clearly beyond the experimental error. The ultraviolet absorption spectra of the same proteins were studied by Smith & Coy (172). The data obtained appeared to be readily interpretable in terms of the content of aromatic amino acids in these proteins.

## Substances Involved in the Blood Clotting Process

Understanding of the chemistry of blood clotting, although still very incomplete, has been greatly advanced by the separation of purified components from plasma, and the study of their interaction. Especially notable is the separation of apparently pure bovine prothrombin by Seegers, Loomis & Vandenbelt (173), already mentioned in last year's review (169). Large quantities of human fibrinogen have been separated in Fraction I of human plasma. The protein in this fraction is, however, only about 65 per

TABLE III

AMINO ACID COMPOSITION OF HUMAN PLASMA PROTEINS (3, 13)

Expressed in gm. per 100 gm. protein

| Constituent | Albumin | Globulin: Gamma | Globulin: Beta | Globulin: Alpha | Fibrinogen |
|---|---|---|---|---|---|
| Total N | 15.95 | 16.03 | 15.24 | | 16.9 |
| Total S | 1.96 | 1.02 | 1.32 | | 1.26 |
| Free Alpha Amino N | 0.18 | 0.11 | | | |
| Amide N | 0.88 | 1.11 | | | |
| Glycine | 1.6 | 4.2 | 5.6 | 3.1 | 5.6 |
| Alanine | | | | | |
| Valine | 7.7 | 9.7 | 7.0 | 5.2 | 4.4 |
| Leucine | 11.0 | 9.3 | 8.2 | 14.2 | 7.1 |
| Isoleucine | 1.7 | 2.7 | 5.0 | 1.7 | 4.8 |
| Proline | 5.1 | 8.1 | 7.1 | 4.7 | 5.7 |
| Phenylalanine | 7.8 | 4.6 | 4.7 | 4.6 | 4.2 |
| Cysteine | 0.7 | 0.7 | | | 0.4 |
| Half-Cystine | 5.6 | 2.4 | 3.4 | 1.5 | 2.3 |
| Methionine | 1.3 | 1.1 | 1.7 | 1.4 | 2.5 |
| Tryptophane | (0.2) | 2.9 | 2.0 | 1.9 | 3.3 |
| Arginine | 6.2 | 4.8 | 6.8 | 7.7 | 7.9 |
| Histidine | 3.5 | 2.5 | 2.8 | 2.8 | 2.8 |
| Lysine | 12.3 | 8.1 | 6.6 | 8.9 | 8.3 |
| Aspartic Acid | 10.4 | 8.8 | 9.8 | 9.0 | 13.6 |
| Glutamic Acid | 17.4 | 11.8 | 14.5 | 21.6 | 14.3 |
| Serine | 3.7 | 11.4 | 7.1 | 5.0 | 9.2 |
| Threonine | 5.0 | 8.4 | 6.1 | 4.9 | 6.6 |
| Tyrosine | 4.7 | 6.8 | 6.0 | 4.5 | 5.8 |
| Hydroxyproline | 0 | | | | 0 |

Albumin, Fraction V, #42.
Gamma Globulin, Fraction II-1, #36.
Beta Globulin, Fraction III-22 beta, #L-421.
Albumin = 1%, gamma = 1%, Alpha = 7%, $Beta_1$ = 82%, $Beta_2$ = 9%
Ash = 8.13%. Values on ash free basis.
Alpha Globulin, Fraction IV-1, Run 146.
Alpha = 94%, Beta = 6%, Lipids = 35.2%.
Values on lipid free basis.
Fibrinogen, Fraction I, #81 RI, 87% clottable.

cent clottable with thrombin; preparations which are approximately 98 per cent clottable are readily obtained by further purification (174). Prothrombin and thrombin, as obtained from Fraction III-2 of human plasma, (147, 175) are still very far from pure, as compared with the best bovine thrombin preparations of Seegers. However, the amount of protein present in such a thrombin preparation is very small, compared to the amount of fibrinogen it is capable of clotting, and its potency is more than sufficient for all clinical and many experimental uses.[9] Ferry & Morrison (177) have carried out a very extensive study of the clotting of fibrinogen by thrombin, systematically varying the concentrations of the reactants, pH, ionic strength, and other variables. Alterations in these variables affected not only the rate of the reaction, as judged, for example, from the clotting time, but also the properties of the clot formed. In general, two extreme types of clots were observed. One type, which is readily obtained at pH values near 6.3, and at moderately low ionic strength and thrombin concentrations, is opaque, doughy, nonfriable, not very adhesive, and displays a very marked tendency to syneresis, with extrusion of liquid. The other extreme type, readily formed at pH values above 7, is transparent and very friable, and does not synerize. Its tensile strength and maximum elongation are low, because of its friability. The transparent clot is termed a "fine" clot by Ferry & Morrison, whereas they call the opaque type a "coarse" clot. They interpret the different qualities of the clots by assuming that two different kinds of reaction are involved in the formation of the clot—first, a process, involving presumably the formation of covalent bonds between different fibrinogen molecules, giving both end to end association and also a certain amount of cross linkage. This is accompanied by a secondary tendency to lateral association of the elongated fibrin chains, due not to covalent bond formation, but to less specific attractive forces, which tend to cause side by side

[9] In the conversion of human prothrombin from Fraction III-2 to thrombin, it is important that thromboplastin of human origin be employed, and preparations made from human placenta have been used for this purpose (175). The thromboplastins of human placenta and lung have now been prepared in purer form than previously, by Chargaff (176), employing centrifugation at 31,000 *g*. The placental material contains about 40 per cent of various lipids, and in the ultracentrifuge gives a principal component for which $s_{20}=270$ S. As a thromboplastin, though highly active, it is somewhat less potent than the preparation previously obtained by Chargaff from beef lung.

aggregation of the long chain molecules first formed. Increase of net charge on the fibrinogen molecules, which results from increase of pH, increases the intermolecular repulsive forces, and thereby decreases the tendency to this secondary lateral association. The "fine" type of clot is assumed to be due to the formation of a relatively loose, open network, the elements of which do not coalesce into larger aggregates because of these repulsive forces; while the opaque "coarse" clots are formed under conditions which favor further alignment and aggregation of the molecules. The general process of clot formation appears to conform qualitatively with the theory of gel formation developed particularly by Flory (178, 179, 180) and by Stockmayer (181) to explain the gelation accompanying polymerization in certain high polymer systems formed by reactions involving components with more than two reactive functional groups per molecule (182). The theory indicates that the solution should set to a gel at a definite critical point, when a certain proportion of the reactive groups has undergone reaction; this proportion depends on the number of reactive functional groups per molecule. The number of linkages formed at the gel point, at which the solution just begins to show rigidity, is very much less than the number which can be formed as the reaction proceeds further. Ferry & Morrison, indeed, demonstrated by quantitative rigidity measurements, that fibrin clots show a progressive increase in rigidity and—at pH near 6.3—also a progressive increase in opacity, long after the first appearance of a definite clot. Moreover, the amount of clottable protein formed, as determined directly by washing out soluble protein and salt, and weighing the dry clot residue, increases slowly to a maximum value, which is not obtained until long after the beginning of clot formation. Thrombin functions as a catalyst, and does not enter as an essential component into the structure of the clot. One gram of pure thrombin can bring about the conversion of more than $10^5$ gm. of fibrinogen to fibrin; and over a wide range the amount of fibrin formed at the completion of the reaction is independent of the amount of thrombin added.

Ferry & Morrison proceeded to prepare fibrin in the form of an elastic film (183), using the opaque "coarse" type of clot as the basis of the preparation. Such a clot, prepared in the form of a broad, thin sheet, from human fibrinogen and thrombin solution, was allowed to synerize and then subjected to moderate pressure

in order to remove water still further. These films could be made in a wide variety of thicknesses and protein concentrations, so that the mechanical strength, and other related properties, could be varied over a considerable range. Detailed studies of tensile strength, maximum elongation, and elastic properties in general, were carried out. The properties of these films can be markedly modified by heat treatment (184), which is thus employed for the double purpose of sterilizing the films, and at the same time modifying their mechanical and chemical properties in order to adapt them for specific purposes. Film, sterilized by steam for twenty minutes at 121° C., has a higher tensile strength, lower swelling in acid and alkali, lower permeability, greater resistance to enzymatic digestion, and higher affinity to dyes than unmodified film. All the observed changes in physical properties due to heat may be explained by formation of new intermolecular cross linkages. Very prolonged heating of steam treated film results in breakdown of the structure, with decreased tensile strength, decreased resistance to trypsin digestion, and changes in other properties.

Heat sterilized fibrin film has found extensive clinical use as a dural substitute, (185, 186) since it does not give rise to adhesions when placed over brain tissue, but is slowly absorbed over a period of four to six months, being replaced by a neomembrane of fibrous tissue. Fibrin film can also be prepared in the form of seamless tubing (187), which has been found very effective in experimental animals, for arterial and venous anastomosis (188). Fibrinogen can also be prepared in the form of plastics, which are capable of a very wide degree of variation in mechanical and other properties, according to the method of preparation (187).

*Fibrin foam.*—This product (189) is prepared from fibrinogen solution, beaten vigorously in a Waring blender, and treated with thrombin so as to form a porous, foamy, rigid clot. This clot is then cut into pieces, frozen, dried from the frozen state, and finally heat sterilized. It found extensive application during the War as a hemostatic agent, especially in neurosurgery (185, 190, 191, 192) but also in general (192, 193) and dental surgery, when used in conjunction with thrombin solution (135, 185).

*Proteolytic enzymes of plasma and the dissolution of the fibrin clot.*—It was found by Milstone (194) that the dissolution of a clot of human fibrin by the fibrinolysin of the hemolytic streptococcus did not occur if the clot was made from highly purified fibrin; ad-

dition of a protein component present in human plasma was essential for the lysis of the clot. The nature of the process has now been further clarified by the work of Christensen (195), Christensen & MacLeod (196), and Kaplan (197) which shows that the active enzyme is actually formed from a precursor present in normal human plasma, the streptococcal factor serving as an activator which very rapidly converts the enzyme precursor into an active proteolytic enzyme. Christensen & MacLeod have appropriately renamed the streptococcal factor "streptokinase." The active enzyme attacks casein, gelatin, and other substrates, as well as fibrinogen and fibrin. Christensen (198) has recently further clarified the process of enzyme activation which occurs when plasma, or certain plasma fractions, are treated with chloroform. The action of chloroform appears to depend on the removal of an enzyme inhibitor, similar in its action to the inhibitor of crystalline trypsin, discovered by Kunitz & Northrop (199). When the inhibitor is extracted by chloroform, spontaneous activation of the enzyme can proceed even in the absence of an activator such as streptokinase. Christensen has called the enzyme precursor "plasminogen" and the active enzyme "plasmin."[10]

It should also be noted that, in contradiction to some earlier reports, Seegers & Loomis (200) have found that the enzyme, if freed from prothrombin and thrombin, has no tendency to cause clotting of fibrinogen. It does, however, destroy prothrombin (but not thrombin) fairly rapidly. Holmberg (201) has reported that the enzyme breaks fibrinogen down into globulin-like fragments with a molecular weight of about 100,000; however, he was not able to detect digestion of any serum protein other than fibrinogen.

## Osmotic Pressure of Proteins

A major advance in the theoretical treatment of the osmotic pressure of protein solutions has been made by Scatchard (202). His equations serve to relate the osmotic pressure, and the distri-

[10] The name "serum tryptase" which has also been suggested for this enzyme seems inappropriate, for Fruton in unpublished studies on enzyme preparations from the Harvard Laboratories, made by Richert, has found that its specificity differs markedly from that of trypsin. The term "fibrinolysin" which has also been used for the enzyme, is likely to be confusing, partly because the same term was earlier widely used for streptokinase and partly because the enzyme is not specific for fibrinogen or fibrin, but attacks other proteins as well.

bution of diffusible solutes, to the variations of the activities or chemical potentials of the components, as the composition of the solution changes. The precise definition of the components, in systems of this sort, is a matter of major importance if an adequate theoretical analysis of the data is to be carried out; and the foundation for such a treatment, on lines not hitherto explicitly formulated, is laid in this paper. Scatchard, Batchelder & Brown (203) have described a modified type of osmometer, and have reported the results of very detailed studies on osmotic pressure of bovine serum albumin solutions, over a wide range of pH, ionic strength, and protein concentration. They deliberately chose to study solutions containing no buffer except the protein itself, since the theoretical treatment of the data is greatly simplified under these circumstances. Analysis of the distribution of sodium chloride across membranes, in the presence of protein, showed that the logarithm of the ratio of the salt concentration, on the two sides of the membrane, was proportional to the albumin concentration, and inversely proportional to the salt concentration. The simple theory of the Donnan membrane equilibrium, expressed in terms of ion concentrations, proved entirely inadequate to account for the observed results. The observed relations can be explained on the assumption that the albumin molecule shows selective, and rather tight, binding of chloride ions, approximately six ions per albumin molecule. This finding is closely related to other studies indicating the binding of many different types of organic anions by serum albumin, which are discussed later in this review.

A major step in the direction of increasing the accuracy of osmotic pressure measurements has been taken by Jullander (204), who, on the basis of suggestions offered by Svedberg, developed an instrument known as the osmotic balance. In this device, the inflow of solvent, across a semipermeable membrane, is determined by a weighing, instead of the measurement of a length, as in the usual osmometer. The vessel containing the inner solution, which is covered by a semipermeable membrane at the bottom, is suspended from one arm of an analytical balance, and dips into the solvent held in a surrounding container, which rests on an adjustable platform. When solvent passes into this balance through the membrane, the balance sinks until the buoyancy compensates the increased weight. The weight necessary to restore the position of the balance gives approximately the amount of solvent that has

passed through the membrane. For the numerous refinements and precautions involved in the method, the original paper must be consulted. The method promises the attainment of osmotic pressure measurements with an accuracy of 0.001 mm. of water. Jullander's studies were concerned with nitrocellulose, but the same method should be readily adaptable to proteins.

Bull (205) has studied the osmotic pressure of $\beta$-lactoglobulin solutions and gives a value of 35,050, markedly below that of 42,000 reported by Pedersen (206) from sedimentation and diffusion, and the value of 42,020 calculated by Brand (3, 4), from amino acid composition. Bull (207) has also determined the molecular weight of gaseous films of $\beta$-lactoglobulin spread on ammonium sulphate solutions, and concludes that the molecule dissociates into two surface active fractions of molecular weight close to 17,000. In the presence of dilute cupric sulphate, the molecular weight becomes 34,300. The explanation of these discrepancies must await further study, by all available techniques, of preparations made under carefully defined conditions. Important effects of the method of preparation on the properties of $\beta$-lactoglobulin have been shown by Grönwall (208) who found that his preparation, obtained by the method of Sörensen & Sörensen (209) had a solubility in dilute sodium chloride approximately twice as great as that of Palmer (210), prepared by a different method. Grönwall's paper, in its other portions, represents probably the most comprehensive study yet made of the influence of amino acid ions and dipolar ions on the solubility of a protein.[11]

## Electrophoresis

The recent studies of Dole (213), and of Svensson (214), have greatly clarified our understanding of the nature and mobility of the different boundaries, which can be observed in a system containing $n$ ions of different mobilities.

> A system that contains $n$ ions will, in general, form $n$-1 boundaries, whether or not any ions disappear over a boundary. If the system contains $p$ anions and $q$

[11] Further doubts concerning the homogeneity of $\beta$-lactoglobulin have been raised in a very recent note by Li (211), who finds three electrophoretic components at pH 4.8 and at 6.5, although only one at pH 5.3 and 5.6.

However, Bosshardt, Moore & Brand (212) find no sign of electrophoretic inhomogeneity at pH 7.4 and 8.6 in a freshly crystallized but not recrystallized preparation of $\beta$-lactoglobulin. At pH 4.0, homogeneity was also indicated.

cations, there will generally be $p$-1 boundaries with negative velocities, and $q$-1 with positive velocities. A system containing $n$ ions may, however, form less than $n$-1 boundaries under specific conditions (213).

However, the observed mobility of a boundary does not correspond exactly to the mobility of any one individual ion present, but is a function of the mobilities of all of them. Both Dole and Svensson assume, in order to simplify the mathematical treatment, that the relative mobilities for all the ions are constant throughout the system. This assumption can not be exactly correct, but the comparison of the equations derived, using this assumption, with the experimental data, indicates that the errors are not serious, at least in many systems involving proteins and smaller ions. In certain cases, the mobility of a boundary may differ very considerably from that of any individual ion in the system; such boundaries have been termed "false" boundaries by Svensson. In general, the boundary anomalies are least for ions of high refractive increments relative to their total net electric charge. Thus, proteins and other large ions are particularly suitable for electrophoresis without the danger of pronounced boundary anomalies. Svensson concludes, from his analysis, that the buffer ion of the same charge as that of the protein ion being studied should preferably be of rather low mobility.

If no buffer ions slower than the leading (protein) ion are available, both cations and anions in the buffer should be chosen with low mobilities, but it is especially important that the ions of the same charge as the leading ion should be slow (214).

"The refractive index increment given by the leading ion is enlarged by the slower surrounding ions of the same charge; it is diminished by faster ions of the same charge and by ions of the opposite charge" (214). It follows that, in the analysis of blood plasma, especially at low ionic strength, unduly high values are obtained for the amount of the fast moving albumin component, and unduly low values for the slow moving $\gamma$-globulin. These errors, as Dole and Svensson have both mentioned, are diminished at high ionic strength and low protein concentration. Svensson has given examples from his own experimental work. The matter has been studied further by Perlmann & Kaufman (215) and by Armstrong, Budka, & Morrison (216). Both these investigations lead to the conclusion that the true value for albumin concentra-

tion, in human plasma, is 51 to 53 per cent of the total weight of plasma protein, several per cent less than the values given by most previous authors. Armstrong, Budka & Morrison also studied known mixtures of purified plasma protein components, in known proportions. A mixture of equal weights of albumin and $\alpha_2$-globulin showed, at ionic strength 0.05 and moderate protein concentration, an apparent albumin concentration 7 per cent greater than the true value. However at ionic strength 0.3 and 1 per cent protein, the electrophoretic values corresponded almost exactly with those deduced from the amounts of the pure components added. The observed deviations between the true concentration values, and the apparent values obtained by electrophoresis, were always in the direction predicted by Dole's theory, but generally of somewhat greater magnitude, indicating some interaction between the components.

Detailed studies of bovine plasma, along similar lines, were made by Koenig, Perrings & Hogness (217). They found the variation of electrophoretic distribution with ionic strength to be much greater in phosphate than in barbiturate buffers; although even in the latter, the effects of ionic strength were not negligible.

The theoretical studies of Svensson and of Dole appear to be well validated by experiment; and the corrections introduced by their treatment must be considered in all calculations of the concentrations of components from electrophoretic data. However, it is apparent that more specific interactions between proteins and the small salt ions present, as well as protein—protein interactions,also greatly influence the observed results. For many purposes, however, it is unimportant to obtain absolute values of electrophoretic concentrations; what is necessary is to obtain good relative values in a series of studies made under comparable conditions.

In addition to the studies above reported, Svensson (214) has described numerous modifications and improvements in the design of the Tiselius electrophoretic apparatus, and has given an extended discussion of the optical system in which a cylindrical lens and a diagonal slit are employed.

A considerable amount of comparative electrophoretic data, on plasmas of different species, is now becoming available. Deutsch & Goodloe (218) have reported individual electrophoretic runs on a large number of different animal species, which reveal very striking differences, in many cases, from one species to another. Similar

results were published by Moore (219). Detailed data on swine serum and plasma were reported by Koenig & Hogness (220); and on beef serum and plasma by Hogness, Giffee & Koenig (221). It is apparent that there are wide variations in relative proportions of albumin and various globulins from one species to another. However, in many cases, it is still uncertain how much variation may be found between various individuals of the same species. Further work on this point will be awaited with interest.

Chanutin & Gjessing (222) have studied in dogs the effects of various types of injury on electrophoretic composition of the serum proteins. The albumin concentration decreased and the $\alpha$-globulin increased. Also sharp spikes appeared in the $\alpha$- and $\beta$-areas of the electrophoretic diagram. Dogs injured with $\beta$-chloroethyl vesicants (223) showed somewhat similar changes. The most marked effects were seen as a decrease of albumin and increase of $\alpha$-globulin after exposure of the body to bis ($\beta$-chloroethyl) sulfide vapor.

The use of electrophoresis in medicine has been reviewed by Stern & Reiner (224). The separation of proteins by the electrophoretic convection technique of Nielsen & Kirkwood (225) suggests new possibilities in preparative electrophoresis, although as yet nothing approaching quantitative separation has been achieved by this method.

## Binding of Inorganic and Organic Ions by Serum Albumin

It has been recognized, for many years, that proteins combine with many organic ions, such as those of dyes, the bonds formed being readily broken again and apparently depending on both electrostatic and Van der Waals forces. Many of the studies in this field have been reviewed by Steinhardt, (226). Several recent studies have been particularly concerned with the unusual properties of serum albumin. A comprehensive investigation of the interaction of anions with many fatty acids has been carried out by Boyer, Lum, Ballou, Luck & Rice (227). Interaction between fatty acid anions and the protein is revealed by changes in thermal stability and viscosity, and by ultrafiltration studies, which give unmistakable evidence of binding of the anion to the albumin. The effects increase rapidly with the length of the hydrocarbon chain up to the $C_7$ and $C_8$ carboxylate anions; at very low anion concentra-

tions, the higher members of the series up to $C_{12}$ are relatively even more effective than the lower members at the same concentration. The effect on thermal stability is so great that concentrated serum albumin, at pH near 7, in the presence of .04 *M* sodium caprylate, may be heated at 60° C. for a period of 10 hours, or even considerably longer, without any significant turbidity appearing in the solution, and without evidence of denaturation of the protein. To be an effective stabilizer, a substance must apparently contain an anionic group, with a fairly large nonpolar side chain, The presence of a hydroxyl group, or of a second carboxyl group, in the fatty acid anion definitely decreases its stabilizing action. Sulfate or sulfonate anions, with an attached hydrocarbon chain, exerted stabilizing effects similar to those of carboxylate anions of the same chain length. Cationic groups, with similar alkyl residues attached, on the other hand, markedly decreased thermal stability of the albumin (227). Scatchard, Strong, Hughes, Ashworth & Sparrow (228) studied some of these, and also other stabilizing agents; the sodium salt of acetyltryptophane proved to be especially effective, being comparable with sodium caprylate in its heat stabilizing action. Indeed, 25 per cent solutions of human serum albumin, containing 0.04 *M* sodium acetyltryptophanate, have now been prepared for clinical use on a fairly large scale. The high heat stability of this preparation permits it to be sterilized by heating at 60° for ten hours, as with caprylate, thereby destroying not only any bacteria that may have survived the previous filtration, but also presumably most, or all, of the viruses, which might conceivably contaminate such preparations.

It was found by Boyer (229) that caprylate, even at low concentrations, protects serum albumin from denaturation in urea or guanidine hydrochloride, provided the concentration of the latter was not too high. More extensive studies were made by Boyer, Ballou & Luck (230). Stabilization against urea and guanidine hydrochloride increased with the chain length of the anion. Addition of carpylate to albumin solutions previously denatured by 6 *M* urea, resulted in a prompt and well marked viscosity decrease. Apparently, the unfolding of the denatured molecule was at least partially reversed by the caprylate.

Not only saturated, but also unsaturated, fatty acid anions are bound by albumin. Davis & Dubos (231) found that albumin has a very powerful affinity for the oleate anion and will remove small

amounts of it almost quantitatively from solution, a finding which proved to be of major importance in preparation of culture medium for the growth of the tubercle bacillus. The albumin removed the oleate and permitted growth if the medium was suitable in other respects. Studies of binding capacities indicated that one molecule of albumin combined quite firmly with approximately six of oleate anions, and could combine rather more loosely with about three more. (232).

Quantitative studies of the equilibria between serum albumin and certain dye anions, such as those of azosulfathiazole, methyl orange, and Orange I and II, have been made by Klotz and his coworkers. Klotz, Walker & Pivan (233) studied albumin solutions equilibrated against the protein-free solvent, across cellophane membranes. Analysis of the outer fluid permitted calculation by difference of the amount of dye anion present in the inner fluid, and hence of the amount bound by the protein. The limiting binding capacity appeared to be about 22 groups of the added ion per mole of albumin, at pH 5.7, a markedly higher value than that found for the oleate anion. Studies of the absorption spectrum of these dyes also indicated definite combination when bovine serum albumin was added (234), whereas bovine $\gamma$-globulin and gelatin produced no effect whatever on the absorption spectrum. Addition of other acid anions, such, for instance, as succinate or *p*-aminobenzoate, in the presence of azosulfathiazole or of Orange I and Orange II, showed that the dye anion could be partially and reversibly displaced by the added acid; that is, that the two anions were competing for the same groups on the albumin molecule. Fieser & Heymans (235) have shown a similar, and very striking, capacity on the part of certain naphthoquinone derivatives to combine with serum albumin—a property not yet found to be possessed in significant degree by any other protein.

The capacity of serum albumin to combine with anions is not restricted to the large, organic anions hitherto discussed. The osmotic studies of Scatchard, Batchelder & Brown (203), on serum albumin in the presence of sodium chloride at different pH values, indicate that albumin combines with one or both of the ions of the salt, and there is strong reason to believe that it combines preferentially with chloride, rather than with sodium, with which it may not combine at all. The number of chloride ions bound was estimated as six or seven per mole of albumin, corresponding closely

to the number of oleate or stearate ions, which can be firmly bound by the serum albumin.

It should be emphasized that all these reactions take place readily on the alkaline side of the isoelectric point of serum albumin; the protein and the dye or other anion combine, even though both carry a negative charge. There is reason for believing, however, that positively charged groups in the albumin molecule, such as those of lysine or arginine, or any free $\alpha$-amino groups that may be present, play a particularly important part in the observed combination. Indeed, the phenomena appear inexplicable, unless both electrostatic and Van der Waals forces are assumed to be operative in the observed binding.

## Nucleoproteins

Pollister & Mirsky (236) have given a detailed report of their studies on the nucleoprotamine of trout sperm, which can be extracted completely with 1 *M* sodium chloride; it is precipitated in long, fibrous strands on diluting to 0.14 *M* salt. On dialysis in molar sodium chloride, the nucleic acid-protamine complex dissociates, and the protein component passes through the membrane. The nucleoprotamine is estimated to make up 91 per cent of the lipid-free mass of the sperm nucleus. Mirsky & Pollister (237) have studied the properties of a desoxyribose nucleoprotein complex, obtained by them from a great variety of animal cells, and from some plant and bacterial cells; they denote this complex as chromosin.[12] Extensive evidence is given that all chromosins are derived from cell nuclei, and that the nucleic acid component contains very little or no ribose nucleic acid. In addition, chromosins contain two protein components: histone, which can be separated by treatment of the complex with 0.2 *N* HCL, and which contains practically no tryptophane; and a nonhistone protein, of lower nitrogen content, not split off by the acid treatment, and containing at least 0.8 per cent tryptophane. Chromosins are to be regarded as a general class of associated complex molecules; typical chromosins have been prepared from isolated chromosomes.

Studies on the protein component of thymus nucleohistone (239) show that it can be separated from the nucleic acid compo-

[12] Not to be confused with the so-called "chromosomin" of Stedman & Stedman (238).

nent by dialysis in molar sodium chloride. The separated protein is probably polydisperse; it has a molecular weight of 69,000 to 79,000 by sedimentation and diffusion.

## Muscle Proteins

The dramatic developments in this field, resulting from the work of Szent-Györgyi and his school, have been discussed in previous reviews (169, 240). Here we can only note a few advances, such as the important electron microscope studies by Hall, Jakus & Schmitt (241) of striated muscle and myosin. The myofibrils are composed of bundles of myosin filaments ranging in width from about 50 to 250 Å. They extend continuously through the isotropic and anisotropic bands in both the extended and contracted state. On extraction by salt solution at pH near 8 (242), the myosin filaments obtained have fairly uniform widths, but highly variable lengths, in general below 15,000 Å. This polydispersity is in accord with previous data on double refraction of flow (243).

Bailey (244) has prepared a new asymmetric protein from the fibrillar fraction of skeletal and cardiac muscle, which he denotes as tropomyosin. The molecular weight is near 100,000. The salt free solution, near pH 7, is very viscous and shows strong double refraction of flow. The amino acid composition of tropomyosin is similar to that of myosin, but it is higher than myosin in the content of lysine and of free ionic dicarboxylic acid groups. Recently, Bailey has succeeded in crystallizing tropomyosin. The crystals are unusual in that they are composed of water to the extent of 90 per cent by weight.[13] In recent studies on actin and myosin, Bailey has found that the sulfhydryl groups of myosin, but not of actin, are essential for actomyosin formation.

No further attempt will be made here to discuss other important investigations proceeding in the field of muscle proteins, since present developments are so rapid that the time seems hardly ripe for evaluating the results. The attention of the reader should be called, however, to the symposium on fibrous proteins held by the Society of Dyers and Colourists in May, 1946, at which a number of important x-ray, analytical and physico-chemical studies, especially on wool keratin, were discussed.[14]

[13] Bailey, K., Personal communication.

[14] A brief summary of the proceedings is given in *Nature*, **158**, 473–76 (1946) The proceedings of thisconference have now been published in a monograph (cf. 66).

## Bacterial Toxins

Within the past year, the isolation and crystallization of two bacterial toxins in apparently pure form has been announced. Pillemer, Wittler & Grossberg (245, 246) described the crystallization of tetanus toxin, using fractionation with methanol at $-4°$ to $-8°$, by methods analogous to those employed in the ethanol fractionation of plasma (132). The crystals contained 50 to $75 \times 10^6$ mouse minimal lethal doses per mg. nitrogen. Activity remained constant through several recrystallizations, and the product gave positive tests with protein reagents. Preparations of almost equal potency had previously been described by Pickett, Hoeprich & Germain (247).

Two different groups of workers, both at Camp Detrick, Maryland, have obtained crystalline toxin preparations from *Clostridium botulinum* Type A (248, 249). The methods used by the two groups differ widely in detail, but both rest largely on the classical procedures of protein chemistry, especially on salting out with sodium or ammonium sulfate. The product contains $220 \times 10^6$ mouse minimal lethal doses per mg. nitrogen, being undoubtedly the most powerful poison yet known; its isoelectric point is at pH 5.6; it is insoluble in distilled water, but soluble in 0.9 per cent sodium chloride at this pH (249). It is homogeneous, both electrophoretically (248, 249) and in the ultracentrifuge (250); $s_{20} = 17.3$ S and $D_{20} = 2.14 \times 10^{-7}$ cm$^2$/sec., giving a molecular weight near 900,000. From diffusion and viscosity measurements, a molecular weight near 1,100,000 was estimated (251).

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# MINERAL METABOLISM

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The general subject of mineral metabolism was last treated in the *Annual Review* in 1945 (1). Since that time several other reviews of certain phases of mineral metabolism have appeared. Russell (2) has presented an excellent summary of mineral deficiencies and excess in pastures. Holmes (3) has dealt with the recent information on the calcium requirements during growth. Several reviews of trace elements with particular reference to plants have been collected and published as a symposium (4). An interesting discussion on the biological function of these elements has been presented by Baudisch (5). A series of papers dealing with their relations to health, presented before the Nutrition Society, has been published (6). The worker in the field of trace elements will find the book by Sandell (7) on the colorimetric determination of traces of metals a very useful source of technics. Two excellent short reviews of various aspects of mineral metabolism have appeared in *Nutrition Reviews* (8). Within the space limitations, the present review covers papers which have appeared in 1945 and 1946, with emphasis on the nutritional aspects of mineral metabolism.

*Phosphorus and calcium.*—Recently several papers have appeared reporting studies of the availability of phosphorus in phytin or phytic acid. Spitzer *et al.* (9) found that for the rat the phosphorus of soybean oil meal of which 58 per cent was in the form of phytin or phytic acid was readily available for growth and bone formation. In a subsequent publication (10) evidence was presented that soybean oil meal phosphorus was available in a ration containing no phytase. The detection of phytase activity in the mucosa of the small intestine of rats which confirmed previous findings of others, suggests that the phytin or phytic acid phosphorus of the soybean oil meal was made available by the action of intestinal phytases. In addition to the rat, phytase activity was observed in the mucosa of the small intestine of the chicken, pig, and cow. Boutwell *et al.* (11) studied the availability to rats of the phosphorus of a diet in which 85 per cent of the element was in the

form of phytic acid and its salts. In the absence of vitamin D the phytin phosphorus of wheat bran was poorly utilized, a finding which is in keeping with earlier reports of others. An adequate intake of vitamin D increased the utilization, making it nearly equal to that of inorganic phosphorus as measured by bone ash. It was also observed that the presence of phosphorus-splitting enzymes in the diets did not alter the apparent availability of phytin phosphorus.

The so-called rachitogenic effect of oatmeal still remains an enigma. Cruickshank *et al.* (12) have published a study bearing on the general problem. Human adults fed a diet containing a large amount of oatmeal showed an almost complete disappearance of phytate-phosphorus during its passage through the intestine, when the calcium intake approached the requirement. These authors feel, therefore, that phytate-phosphorus can hardly be considered a factor seriously interfering with the absorption of dietary calcium.

The short supply of highly available phosphorus supplements for farm animals during the war stimulated the search for other phosphorus compounds that might be used to supplement animal rations. A cooperative study of the effectiveness of ten different samples of phosphates as sources of phosphorus for the formation of bones in chicks was reported by Bird *et al.* (13). The phosphorus sources included six defluorinated superphosphates and one each of defluorinated rock phosphate, phosphate slag, $\beta$-calcium pyrophosphate, and vitreous metaphosphate. When bone ash was used as the criterion of the availability of the phosphorus, one sample of defluorinated superphosphate was almost completely unavailable. The phosphorus of the other five samples of superphosphate was partially available but less so than that in bone meal or tricalcium phosphate. The availability of phosphorus in the defluorinated rock phosphate, phosphate slag, and vitreous calcium metaphosphate samples was intermediate between the phosphorus in the superphosphates on the one hand, and in bone meal and tricalcium phosphate on the other. Calcium pyrophosphate was totally unavailable or nearly so. The solubility in 0.25 per cent hydrochloric acid at 38° C. was found to be a quick approximate measure of the available phosphorus. Each of the supplements fed in one experiment appeared to have a detrimental effect on growth of chicks when fed at levels equivalent in phosphorus content to 2 per cent of bone meal. The results obtained in this experiment

would have been more valid had the phosphorus content of the diets been lower and thus closer to the minimum requirements of chicks. Care was exercised by Matterson *et al.* (14) to feed diets in which the total phosphorus content was near the minimum requirement of chicks. In this study the phosphorus of raw and fused rock phosphate was as well utilized by chicks as the phosphorus of tricalcium orthophosphate. The phosphorus of calcium metaphosphate was utilized to a lesser extent. Singsen & Scott (15) found that the phosphorus of tricalcium orthophosphate and bone meal were as available for bone formation in the chick as monosodium orthophosphate.

A study of the availability to rats of calcium and phosphorus in defluorinated phosphates was reported by Ellis *et al.* (16). Defluorinated rock phosphate prepared by the fusion process compared favorably with bone meal or calcium phosphate as a calcium and phosphorus carrier. The phosphorus of phosphate slag was rated fair to good in availability. Various commercial defluorinated superphosphates were given a similar rating. Calcium $\beta$-metaphosphate, $\beta$-pyrophosphate, and to a less extent $\gamma$-pyrophosphate were found to be relatively unavailable, but both the $\alpha$- and $\beta$-ortho forms of tricalcium phosphate were highly available. A good correlation was found between solubility in dilute hydrochloric or citric acid and the availability ratings for the individual products.

Shrewsbury & Vestal (17) reported that a defluorinated phosphate and a rock phosphate were inferior to steamed bone meal and superphosphate in producing high-quality bone in growing swine. For bred sows and gilts, steamed bone meal was only slightly superior to the other two products.

An extended study of the monthly protein and phosphorus contents of two important range grasses—black grama and mesa dropseed grass—was reported by Watkins & Knox (18). The peak values for both protein and phosphorus were shown to occur during either September or October, and it was at this time only that the phosphorus requirements of beef breeding cattle were met by the grasses. Beeson *et al.* (19) found that a ration containing 0.14 per cent of phosphorus is on the borderline for adequately maintaining a normal phosphorus balance in pregnant ewes. Rations containing 0.16 to 0.19 per cent of phosphorus proved adequate.

A method has been described for determining inorganic phosphorus in the presence of labile phosphate esters (20).

Zucker *et al.* (21, 22) have provided additional evidence that

necrosis, hemorrhage, and epithelial hyperplasia of the antral mucosa of the stomach of the rat are an early manifestation of calcium deficiency. Such lesions have not heretofore been associated with this deficiency.

Campbell & Sherman (23) published a study showing that increasing the calcium content of a basal diet, made up of a whole milk powder, wheat, and salt, from 0.34 per cent to 0.64 per cent did not result in any significant increase in growth, reproductive efficiency or life span in rats. The interesting question of the effect of excess dietary calcium on tissue calcification and longevity has been studied by Shields & Mitchell (24). The various levels of dietary calcium employed were found not to affect appreciably the rate of growth of rats. The data suggested a slight tendency for somewhat greater tissue calcification on the highest calcium regime studied (1 per cent).

Greenberg (25) has presented evidence from isotope studies that vitamin D has a direct action on the mineralization of bone in rachitic animals, in addition to promoting healing indirectly by increasing the absorption of calcium.

The calcium requirements of growing ranch-raised silver foxes have been studied by Harris *et al.* (26). Sobel *et al.* (27) have reported studies of the composition of bone in relation to the blood and diet. It was found that the composition of the bone is related to the phosphorus/carbon dioxide ratio, the calcium/phosphorus ratio, and the calcium-phosphorus product in the blood serum. An excellent study of the variations in blood levels of calcium and inorganic phosphorus in a large number of Hereford cattle was published by Payne *et al.* (28). The potassium and calcium contents of gastric and colon carcinomas and colon papillomas has been reported (29, 30). Masters *et al.* (31) in a study of the chemical composition of human thoracic aortas found that the most outstanding changes with age were a marked increase in the calcium, phosphorus and cholesterol contents. A rather intensive study has been made of the ionized serum calcium in children (32). An inverse relationship has been noted between the dietary level of calcium and the rate of thymic involution in rats (33).

*Potassium, sodium, and chlorine.*—Ruegamer *et al.* (34) have described the effects of a dietary deficiency of potassium in dogs. The symptoms consisted of "stiff neck" which progressed to a general paralysis, discolored teeth, and apparently a hemoconcentra-

tion. The conflicting results and interpretations regarding the signs of potassium deficiency in the rat have been considerably clarified by the studies of Kornberg & Endicott (35). These authors have carefully described the symptoms in rats fed diets very low in potassium as being prompt failure in growth, edema, and multiple lesions in many tissues and death. A dietary potassium level of approximately 0.17 per cent appeared to be minimal for optimum growth and prevention of lesions.

A photometric method for the determination of potassium in biological material has been described by Pereira (36). The author states that this method is capable of determining amounts as low as approximately 8 $\mu$g. with an error less than 3 per cent. The potassium and sodium concentration of the blood cells and sera of eight normal persons has been reported (37).

The chemical and pathological changes due to a chloride deficiency in the rat have been further characterized by Cuthbertson & Greenberg (38). The symptoms noted were a decrease in the rate of growth, reduced chloride content of the blood serum, and many other tissues and extensive kidney damage in the late stages of the deficiency.

The necessity of feeding supplemental salt to growing pigs fed rations containing no feeds of animal origin has been shown by Vestal (39, 40).

*Iron.*—Evidence is accumulating that the intestinal mucosa actively participates in the absorption of iron. Copp & Greenberg (41) using a new radioactive iron ($Fe^{55}$) of higher specific activity than the older $Fe^{59}$, have published an excellent study of the absorption and excretion of this element in the rat. Following parenteral administration of $Fe^{55}$, no significant excretion was noted in the bile, urine, or feces. Iron depleted rats were shown to absorb more iron than the controls. This supports the thesis of Whipple that "Absorption of iron is dependent on the need of the body for iron." The internal metabolism of iron is the subject of another paper by Copp & Greenberg (42). By the injection of $Fe^{55}$ it was shown that the element is taken up in large part by the bone marrow for the production of hemoglobin, that the liver is the chief site of iron storage, and that there is a rapid rate of turnover of the element in the small intestine and the bone marrow.

Granick (43, 44) has studied the increase of the protein, apoferritin, in the intestinal mucosa following the feeding of iron.

Ferritin was just detectable in the duodenal mucosa but rarely elsewhere along the intestinal tract. When ferrous iron was fed the ferritin was found to increase markedly, especially in the duodenal and jejunal regions. It was concluded that "These data, when considered together with radioactive iron absorption studies, suggest that ferritin in the mucosa is concerned with the regulation of iron absorption and represents the primary factor for the mucosal block. The feeding of iron leads to the increase in the concentration of the specific protein apoferritin, which appears in the form of ferritin." It would thus appear that the iron-protein complex, ferritin, of the mucosa reaches an equilibrium with the iron reserves of the body, thus governing the absorption of iron from the digestive tract. Evidence has been presented by Vahlquist *et al.* (45) that contrary to the general opinion, iron is also absorbed from the stomach, at least in rabbits and man.

Darby (46) has published the interesting observation that certain oral lesions, such as angular fissures and superficial glossitis, may accompany chronic iron deficiencies. Certain of these cases responded to iron therapy alone; others responded to iron administration after they had failed to yield to prolonged treatment with various B-vitamins. This observation supports others indicating that oral lesions, once regarded as specifically due to a deficiency of some members of the B-complex, may be found under a variety of other circumstances.

A rather detailed description of the morphological changes which occur in the red blood cells in iron deficiency anemias has been presented by Schwartz & Flowers (47). Inflammation induced in dogs by the injection of turpentine was shown by Hahn *et al.* (48) to reduce the absorption of iron. This observation may shed some light on the problem of refractory anemias associated with infectious and inflammatory diseases. Hahn (49) has described a simple method for the preparation of colloidal ferrous iron suitable for intravenous injection. Such iron is well tolerated by dogs, in contrast to the low tolerance to ionizable iron injected intravenously. It has been reported by Neary (50) that a molybdenized iron sulphate gives more rapid hemoglobin formation than iron sulphate in patients with an iron deficiency anemia. Heppel & Kornberg (51) have published results showing that the feeding of iron salts to rats prevents the symptoms due to toxic amounts of dietary lead.

The availability of iron from various inorganic salts for the rat was determined by Freeman & Burrill (52). Of the compounds studied ferric chloride was the most effective and sodium iron pyrophosphate the least, with respect to the relative degree of iron retention and hemoglobin regeneration. Data on the biologically available iron in fifteen Hawaiian grown vegetables have been reported by Miller & Louis (53). The available iron ranged from 6 per cent in asparagus to 96 per cent in green soybeans. The dog has been studied by Ruegamer *et al.* (54) as an assay animal for testing the available iron in feeds.

The serum iron in new-born babies was found by Black & Stoker (55) to exceed the serum iron of the mothers, and to range from 67 to 275 $\mu$g. per 100 ml. of serum. In a nicely designed experiment, the blood serum copper and iron and the hemoglobin and red cell volume of the blood of four breeds of dairy cattle have been determined by Matrone *et al.* (56). Serum copper, hemoglobin, and red cell volume was found to increase with age. Serum iron did not vary with age nor were any differences found among the breeds studied.

A method for determining iron in powdered milk using 1, 10-phenanthroline has been described by Pyenson & Tracy (57). A comparison of three methods (*o*-phenanthroline, thiocyanate and titanous chloride) of determining iron in plant ash has been made by Benne & Snyder (58). Ramsay (59) has published a study of the microvolumetric determination of iron in blood based on microtitration with titanium sulphate. Ruegamer & Elvehjem (60) have described a rapid method for determining iron in enriched bread, without preliminary ashing.

The extensive uses of blood during the war years apparently has stimulated extensive reinvestigations of the technics of determining hemoglobin and the variations encountered (61 to 65). Abbott *et al.* (66) have published the results of studies of hemoglobin values for over 2000 rural school children in Florida. It has been reported by Stein & Carrier (67) that castration or splenectomy of guinea pigs results in a decrease in the red cell count but not in hemoglobin. The injection of liver or iron citrate was associated with a return of the red cell counts to normal.

*Copper.*—Further work has been published relative to the condition known as "swayback" in lambs in Derbyshire, England (68, 69, 70). Previous findings that copper is involved have been

confirmed but it appears that a low copper content of the natural herbage is not the entire answer to the problem and a tentative suggestion of a copper depressing factor in the herbage of the trouble areas has been made. The possibility of a copper deficiency area in Aberdeenshire is indicated (71).

A selective craving for licking copper metal by fattening pigs was shown by Braude (72) not to indicate a dietary need for additional copper. This is an interesting example of a depraved appetite which apparently did not mirror a nutritional deficiency.

The excretion and the effect on the level of copper in the blood of human subjects fed copper sulphate has been reported (73). Evidence that ascorbic acid oxidase is a specific enzyme and not simply a loose complex of copper and protein of unspecific nature has been advanced (74).

Cartwright *et al.* (75) have described a method of determining copper in the blood without previous ashing. A modification of the diethyldithiocarbamate method of determining copper in whole milk powder has been published (76).

*Cobalt.*—Additional reports of cobalt deficient areas, notably in Wisconsin (77), Michigan (78), New Hampshire (79), and North Carolina (80) and also in the British Isles (81) and in Scotland (82) have stimulated increased interest in this element which, strangely, appears to be required by ruminants only.

The partition of radioactive cobalt by cows with rumen fistulas has been reported by Comar *et al.* (83, 84). Intravenously injected, labeled cobalt was excreted chiefly in the urine. Approximately 7 per cent appeared in the feces and none was found in the rumen. On the other hand, labeled cobalt introduced directly into the rumen was excreted chiefly in the feces and none was detected in the blood, milk, or saliva, indicating very poor absorption of the element. Sheline *et al.* (85) studied the elimination of labeled cobalt in the pancreatic juice and the bile of dogs. Significant amounts of intravenously injected cobalt were not found in the pancreatic juice but a total of 5 per cent was eliminated by way of the bile.

The apparent nonrequirement of cobalt by simple-stomached animals has been very puzzling, although recent work tends to support the idea. Houk *et al.* (86) fed rats a diet extremely low (0.003 p.p.m.) in cobalt and obtained no evidence of any need. Thompson & Ellis (87) obtained no evidence that rabbits fed a diet containing 0.0024 p.p.m. of cobalt suffered from a deficiency.

Maunsell (88) in a study of New Zealand pastures found no relationship between the cobalt content and the rate of growth of the pasture. Beeson *et al.* (80) noted a seasonal difference in the cobalt content of a reed which is widely consumed by grazing cattle on the Coastal Plain of North Carolina. This paper presents evidence, based on forage analyses, that cobalt may be deficient in many areas in the Atlantic Coastal Plain.

Stanley *et al.* (89) have published further data on the polycythemia induced by feeding cobalt salts. Data have been published on the cobalt content of some New Zealand limestones (90). A new, very sensitive method of determining cobalt in biological materials has been described by Ellis & Thompson (91).

*Manganese.*—Smith *et al.* (92) have described the symptoms caused by a deficient intake of manganese in rabbits. A deficiency of this element interferes with the development of the skeletal system much as it does in the case of poultry. It would appear that the skeletal system of the rat is not as sensitive to a deficiency of manganese as either poultry or rabbits. In an early report Barnes *et al.* (93) were unable to demonstrate any abnormal tibia development in first generation rats raised on a diet low in manganese, although two cases of defective tibias appeared in the offspring of manganese deficient females. More recently Shils & McCollum (94) observed skeletal abnormalities in rats born of manganese deficient females. By use of more rigid technics Amdur *et al.* (95) were able to demonstrate a decrease in bone length, density, breaking strength, and phosphatase activity in first generation rats fed a diet low in manganese. Evidence is thus accumulating indicating a basic but unclarified role of manganese in the development of the osseous system.

A lipotropic action of manganese in the rat has been indicated by the studies of Amdur *et al.* (96). Skinner & McHargue (97) have published data which do not support the concept that manganese is concerned in the synthesis of ascorbic acid by guinea pigs. Reineke & Turner (98) have shown that manganese catalyzes the formation of thyroxine in iodinated casein. The green color frequently obtained when plant tissues are ashed has been shown by Robinson (99) to be due to the presence of manganese.

*Fluorine.*—A review of the problem of fluorine and dental caries has been published by Getting (100). Sodium fluoride has been shown by Shaw *et al.* (101) to reduce the incidence of molar

caries induced in cotton rats by sucrose-containing diets. McClendon & Foster (102) have published evidence that fluorine is required in the diet of rats. If these observations can be confirmed, fluorine must be added to the known list of essential mineral elements.

McClure (103) was unable to observe a correlation between the fluorine content of the drinking water and height, weight, or the incidence of bone fractures in selectees of the Armed Forces. McClure & Kinser (104) observed that the fluorine content of the urine of men and boys increased in proportion to the fluorine content of the drinking water. This relationship is considered to reduce the presumed hazard of cumulative toxic bone-fluorosis from fluorine-containing waters. An improved method for determining fluorine in minerals and bones has been described by Godfrey & Shrewsbury (105).

*Iodine.*—A symposium on the preparation and biological effects of iodinated proteins has appeared (106). Blaxter (107) has published the results of an extensive cooperative study with lactating cows fed iodinated casein. It was found that the feeding of iodinated casein increased the mean milk yield about 22 per cent. Following the cessation of treatment, the milk yield decreased sharply after several days. In spite of the additional feeding of concentrate feeds, the cattle lost weight.

Halverson *et al.* (108) has described a diet which when fed to rats readily produces an iodine deficiency. Supplementing this diet with 2 $\mu$g. of iodine per rat per day resulted in almost complete prevention of the pathological changes in the thyroid glands.

Iodine fractionation studies have been made of human goitrous thyroid glands by Leblond *et al.* (109). It was found that thyroid adenomas were less active than surrounding tissue as shown by a lower iodine content, smaller fixation of administered radioactive iodine and a slower turnover of the element. Spector *et al.* (110) have reported studies of the excretion of iodine by human subjects held at various environmental temperatures. At least 75 per cent of the total iodine lost from the body was excreted in the urine. No evidence was found that a sweating environment increases the iodine requirement. Methods for determining iodine in biological materials (111) and in blood plasma (112) and for determining thyroxine in iodinated casein (113) have appeared.

*Interactions among micronutrient elements.*—One of the interesting recent discoveries in the field of micronutrient ("trace")

elements involved the scouring of cattle on the so-called "teart" pastures in England and the therapeutic effect of copper salts. In continuing their earlier studies, Ferguson *et al.* (114), Lewis (115, 116), and Ferguson (117) observed the high molybdenum content of "teart" pasturage and were able to duplicate the symptoms displayed by cattle on such forage by feeding molybdenum salts. It was further shown that copper sulphate given as a drench prevented and cured the symptoms. More recently Britton *et al.* (118) have shown that a similar molybdenum toxic area is present in California. The symptoms observed in cattle and the preventive effect of copper confirms the findings of the English workers. Further evidence of a molybdenum-copper interaction has been given by Dick *et al.* (119) who observed that molybdenum fed to sheep and cattle results in a reduction of the copper content of the liver even when additional copper was fed. It would thus appear that a high intake of molybdenum in ruminants precipitates a copper deficiency which is prevented and cured by the feeding of additional copper.

Other interactions between micronutrient elements have been observed by others. Smith *et al.* (120) have shown that an anemia induced by feeding zinc salts to rats was counteracted by feeding additional copper salts. Skinner & McHargue (121) have published evidence that arsenic and manganese fed in addition to iron and copper salts permit greater hemoglobin production in rats than iron and copper fed as the only supplements to a milk diet. A recent publication by Moxon *et al.* (122) confirms earlier reports that arsenic alleviates the toxicity of selenium-containing rations. The apparent supplementary action of molybdenum and iron in the treatment of iron deficiency anemia in man has been mentioned. It is to be hoped that further work will clarify and establish the mode of actions of these rather puzzling interactions.

*Other minerals.*—By use of $S^{35}$ it has been shown by Dziewiatkowski (123) that orally fed sulfide sulfur is oxidized to sulphate sulfur by the rat, thereby rendering toxic form of sulfur innocuous. Small fractions of $S^{35}$ were found in all tissues examined including the hair. In a further publication (124) it was shown that sulfide sulphur ($S^{35}$) can be used by the rat for the synthesis of cystine; $S^{35}$ containing cystine was isolated from the hair, liver, muscle, and skin. Evans & St. John (125) have compared several methods for determining the sulfur content of feeds.

A study of the elimination and distribution of selenium in

sheep, fed graded doses of the element, has been published by Rosenfeld & Beath (126). Sheep fed selenium until death occurred excreted less selenium in the urine towards the end, due to kidney injury. The tissue storage of selenium depended on the intake and was highest in the liver and kidneys. A small amount of the element was present in the tissues two months after removal of selenium from the rations. In a further paper (127) the partial protection against selenosis afforded by high protein rations was confirmed. It was determined that symptoms of selenosis appeared when the blood level of selenium ranged from 1.2 to 1.5 p.p.m. and death resulted when the level exceeded 2 p.p.m. In a third paper (128), several chemical changes in tissues following selenium administration were reported.

Inactivation of penicillin by zinc salts has been observed by Eisner & Porzecanski (129). Alexander & Taylor (130) have described an improved dithizone procedure for the determination of zinc in foods.

Three interesting papers concerning vanadium have been published by Bertrand (131). An extended survey of sixty-two different plant species indicated that vanadium is an almost constant constituent of plants, of the order of magnitude of 1 mg. per kg. of dry matter. It was also observed that the poisonous toadstool, *Amanita muscaria*, is a vanadium accumulator (132). Evidence was obtained that *Aspergillus* require vanadium (133). The mineral content—sodium, potassium, magnesium, and calcium—of human epidermis was studied by Suntzeff & Carruthers (134).

*Effects of acid beverages on teeth.*—In 1943 McClure (135) reported that acid beverages and fruit juices, when consumed regularly in place of drinking water, had a destructive effect on the molars of white rats. These findings have been confirmed and extended by a series of studies at the U. S. Naval Medical Research Institute. Restarski *et al.* (136) reported severe destruction of the enamel of rats' molars by allowing the animals to drink a "popular soft beverage" for five days. Similar results were observed with a prepared solution of the same phosphoric acid and sucrose content and the same pH (2.6) The presence of sucrose aggravated the effect of the acid, but the inclusion to 1 to 20 p.p.m. of fluorine decreased the destruction effect. In a later report by Gortner and co-workers (137) etching of the teeth by certain other acids and by orange juice is described. Oxalic acid solutions having a pH as low as 2.1 did not, however, cause damage. In fact, when this acid

or its sodium salt was present in the food or drink the decalcification caused by the acids previously studied diminished or disappeared. Oxalates and oxalate-containing foods were found to produce protective films on the rats' molars. In extending the studies to dogs and monkeys (138) the deleterious effects of acid-sucrose solutions were again observed and the protective effect of fluorine was also noted (dogs). In these various reports the authors emphasize the need for caution in interpreting their results to human practice, particularly because the acid-containing beverages were used as the sole drink. The need for further research is stressed. The authors recognize that acidity is not the entire explanation of the destructive effect and this observation is supported by the finding of McClure & Ruzicka (139) that, even at neutral reaction, citrate ion is destructive to rats' molars. These investigators consider that the action of citrate is due to the binding of the calcium of the dentin and enamel to form a soluble calcium citrate complex.

*Factors affecting mineral content of food crops.*—Some important contributions have appeared regarding the influence of soil and climatic factors on the mineral nutrient content of food crops. Beeson (140) has reported a survey of the occurrence of mineral nutritional diseases of plants and animals in the United States. Maps are presented showing areas of deficiency in terms of crop response and areas in which animal growth and production are limited by the deficiencies of various minerals in the herbage. Beeson (141) has also published a critical review of over 100 recent papers dealing with the effect of mineral supply on the mineral concentration and nutritional quality of food crops.

Important data in this general field have been obtained from a cooperative project carried out by the experiment stations of the Southern States to determine the significant factors affecting the nutritive value of vegetables. A report by Sheets and co-workers (142) deals with the effect of fertilizer, soil composition, and climatological conditions on the calcium and phosphorus content of turnip greens. Using a single variety of turnip and uniform methods of planting, fertilizing, and harvesting, thirty-nine experiments were carried out in thirty-one localities, so planned as to permit a statistical analysis of the data as a whole. The results showed that conditions associated with location caused from thirteen to twenty times more variation in calcium and phosphorus content than did fertilizers. Fertilization with calcium increased its content in the

crop by only 0.03 per cent on the average, although markedly larger increases were noted in certain locations, notably where the soil was very low in this mineral. Applied phosphorus increased plant phosphorus by approximately 0.05 per cent, but decreased calcium by the same figure. Nitrogen fertilization, which had by far the greatest effect in improving yield, decreased calcium content by 0.36 per cent. These studies reveal the dominant influence of location and soil type. They show that a fertilizer treatment may increase the content of one nutritionally important mineral in a crop, and, on the other hand, decrease that of another, and that fertilization for yield may decrease the percentage composition of these minerals. The findings indicate the unreliability of generalizations regarding the effect of fertilization on nutritive values, but they do reveal that nutritionally important recommendations should be possible for specific soils and locations.

The results of a similar cooperative study of the iron content of turnip greens have been reported by Speirs and co-workers (143). While very wide variations in iron content were noted, these variations were influenced more by environmental factors than by soil fertilization. Of the fertilizer treatments, nitrogen had the largest and most consistent effect, resulting in a decrease in iron content. The influence of the place where grown, however, was greater than this effect of nitrogen fertilization.

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# THE CHEMISTRY OF THE HORMONES

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## Adrenal

Venning *et al.* (1, 2) developed a method for the assay of urinary corticosteroid-like substances based upon the glycogen deposition in the livers of glucose treated adrenalectomized mice. A glycogenic unit is defined as the equivalent of the biological activity of 1 μg. of 17-hydroxy-11-dehydrocorticosterone. It was pointed out by these workers that the administration of glucose increased the sensitivity of the test many times. Similar assay methods have also been described by Dorfman *et al.* (3) and Eggleston *et al.* (4) using adrenalectomized mice without glucose treatment. Dorfman *et al.* (5) reexamined the cold test of Selye & Schenker (6) for the bioassay of urinary corticoids and found that the method was not practical for extended routine use.

Two chemical methods for the determination of the urinary corticosteroid-like substances have also appeared. Heard & Sobel (7) introduced a color metric method, based on the reducing sugar-like property of the adrenal cortical hormones, for the quantitative estimation of these steroids; the color was produced by the reduction of phosphomolybdic acid to molybdenum blue in glacial acetic acid. The reaction is given by steroids containing a primary or secondary (but not tertiary), α-ketol grouping, an α, β-unsaturated 3-ketone group, or both. Thus, desoxycorticosterone exhibits the strongest reducing properties. The method has applied to the estimation of urinary neutral lipide soluble reducing substances as an index of adrenal cortical function (8) and reveals a good agreement between results obtained by this chemical method and that by the bioassay technique of Venning *et al.* (1).

The method of Lowenstein *et al.* (9) in determining corticosteroids in urines is based on periodate oxidation of the primary alcohol group at $C_{21}$ which yields one mole of formaldehyde per

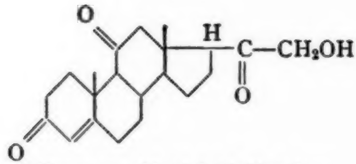
[1] This review includes the following subjects: adrenals, gonads, insulin, and anterior pituitary. Due to the limitation of space, other topics have been omitted.

mole of corticosteroid oxidized; formaldehyde is then determined with chromotropic acid.

When the glycogen deposition test was employed in glucose treated adrenalectomized mice, 11-dehydro-17-hydroxycorticosterone proved to be more than three times as active as 11-dehydrocorticosterone (1). Dorfman *et al.* (10) obtained similar differences in biological potency of these two steroids using both cold protection and liver glycogen tests. On the basis of the cold test employing adrenalectomized rats, the same investigators found that corticosterone and 11-desoxycorticosterone acetate were respectively one-twelfth and one-thirteenth as active as 17-hydroxy-11-dehydrocorticosterone. Pabst *et al.* (11) have applied both the liver glycogen deposition test in adrenalectomized rats and the work performance test of Ingle to certain adrenocortical hormones and found that both tests showed 17-hydroxycorticosterone to be more active than 11-dehydro-17-hydroxycorticosterone and that corticosterone and dehydrocorticosterone were considerably less active than their 17-hydroxy-derivative. 17-hydroxycorticosterone is also shown to be more active in causing an increase of sodium and nitrogen excretions than that induced by corticosterone in acute tests on rats force-fed a high carbohydrate diet (12).

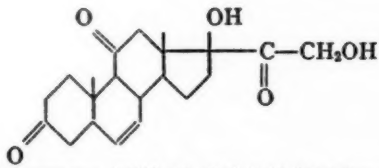
Thatcher & Hartman (13) have described a procedure to prepared a substance highly potent in its ability to cause retention of sodium from adrenal extracts. The procedure involved either fractional solvent precipitation or molecular distillation or chromatographic adsorption. The sodium-retaining activity of the noncrystalline substance was shown to decrease considerably by treatment with hydrochloric acid or acetylation. Its solubility characteristics were quite different from desoxycorticosterone, and its adsorption behavior on a column of alumina also was found to differ from the steroid. In molecular distillation the sodium retaining substance distilled at a higher temperature than does desoxycorticosterone. Furthermore, the substance has no effect on potassium excretion. These workers cast doubt on the existence of desoxycorticosterone as an original constituent in the adrenal gland.

The partial synthesis of dehydrocorticosterone (I) has been achieved by Lardon & Reichstein (14). In the procedure of preparing this steroid it is necessary to prepare 3($\alpha$)-hydroxy-$\Delta^{11}$ cholenic acid.

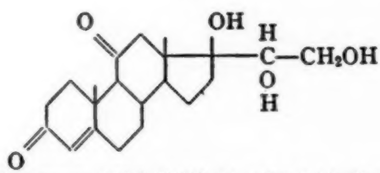


4-Pregnene-21-ol-3,11,20-trione
(dehydrocorticosterone)
I

McKenzie *et al.* (15) have reported the synthesis of this unsaturated acid[2] from desoxycholic acid; the double bond between $C_{11}$ and $C_{12}$ of the steroid has been demonstrated by Gallagher (16). The partial synthesis of 4-pregnene-17(β), 21-diol-3,11,20-trione acetate [II, Compound E of Kendall, Mason *et al.* (17, 18)] and of 4-pregnene-17(β), 20(β), 21-triol-3,11-dione [III, Substance U of Reichstein and von Euw (19)] has been accomplished by Sarett (20) using desoxycholic acid as the starting material.



4-Pregnene-17(β),21-diol-3,11,20-trione
(17-hydroxydehydrocorticosterone)
II



4-Pregnene-17(β),20(β),21-triol-3,11-dione
III

In a preliminary report, Lowenstein & Zwemer (21) described the isolation of an active ketonic steroid from aqueous extracts of

[2] The preparation of 3(α)-hydroxy-$\Delta^{11}$-cholenic acid was first carried out by Wintersteiner et al. (29, 30).

adrenal glands with the empirical formula, $C_{25}H_{34-36}O_9$. The presence of ascorbic acid in this compound was demonstrated. It was further claimed that the activity of the compound is as active or more active than desoxycorticosterone in life maintenance and has similar carbohydrate activity to that given by dehydrocorticosterone.

Tipton *et al.* (22) observed that adrenalectomy in rats caused a decrease in the activities of both succinoxidase and cytochrome oxidase enzyme systems in the livers and that the decrease of these enzymic activities could be prevented by the treatment with adrenal cortical extract. Folley & Greenbaum (23) studied the effects of adrenalectomy and of treatment with adrenal cortical hormones on the arginase activity of the liver and mammary gland and the alkaline phosphatase of the mammary gland and kidney in lactating rats maintained either on a normal diet or on a high protein diet. As previously reported by Fraenkel-Conrat *et al.* (24), adrenalectomy decreased the liver arginase levels, restoration of which was shown to be effected by treatment with 17-hydroxy-dehydrocorticosterone acetate. Moreover, Folley & Greenbaum found that desoxycorticosterone acetate was active in restoring the liver arginase activity of adrenalectomized rats; the effect of this steroid was greater than that of 17-hydoxy-11-dehydrocorticosterone or 11-dehydrocorticosterone if a high-protein diet was used. In contrast, neither desoxycorticosterone nor 11-oxygenated steroids elevated the arginase content of the mammary gland after adrenalectomy. On both diets, adrenalectomy reduced the kidney alkaline phosphatase but had no significant effect on the mammary gland phosphatase; the kidney phosphatase activities could be elevated almost to normal levels by desoxycorticosterone irrespective of diet, and by 11-oxygenated steroid hormones on the high protein diet only. In an earlier paper, Kochakian & Vail (25) reported that the kidney alkaline phosphatase of rats was not altered by adrenalectomy, but the acid phosphatase decreased and was restored to the normal level on administration of adrenal cortex extract.

Conway & Hingerty (26) have analyzed various constituents in skeletal muscle of rats after adrenalectomy. When compared with similar analyses on control rats, they found that concentrations of potassium, magnesium, phosphocreatine and glucose-1-phosphate were elevated, whereas sodium, glucose-6-phosphate

and fructose-1,6-diphosphate decreased. It may be recalled, in relation to the effect on the glucose-1-phosphate, that Shumann (27) and Verzar & Montigel (28) have shown that adrenalectomy in rats caused an inhibiting effect on the phosphorylase action in excised muscle.

## Gonads

*Androgens.*—Several methods have been reported for the determination of 17-ketosteroids in urinary extracts including androsterone, dehydro-iso-androsterone, etiocholanol-3($\alpha$)-one-17, isoandrosterone, androstenone-17 as well as other androstane derivatives. Barnett *et al.* (31) have modified the original polarographic technique of Wolfe *et al.* (32) to estimate these steroids in the urine following the suggestion of Werthessen & Baker (33). They introduced an oxidation step with potassium permanganate in aqueous dioxan to destroy interfering materials under conditions which do not affect 17-ketosteroids. By this method, these workers found that normal young men excreted an average of 15.7 mg. neutral 17-ketosteroids per twenty-four hours with a standard deviation of 2.47 mg.

Dingemanse *et al.* (34) developed a chromatographic colorimetric method for clinical use in the determination of urinary 17-ketosteroids. A column of aluminum oxide standardized according to Brockmann was used; benzene or benzene-alcohol solutions were employed as eluates. The 17-ketosteroids content of each eluate was estimated separately with the Zimmermann reaction as modified by Callow *et al.* (35). Thus, it was shown that normal urine contains at least seven different 17-ketosteroids and that urines from patients with adrenal tumors and with hirsutism or virilism give entirely different patterns.

The metabolism of dehydroisoandrosterone in male rabbits has been found to be different from that studied in human subjects. Mason & Kepler (36) were unable to isolate the unchanged ketosteroid in human urine while Hoffman & Desbarats (37) found that a measurable amount of dehydroisoandrosterone was recovered from the urine of the male rabbit. It may be recalled that in the urine of a man with pituitary insufficiency some unchanged dehydroisoandrosterone could be isolated (38). Following the administration of dehydroisoandrosterone to normal human subjects, the following ketosteroids are obtained in the urine:

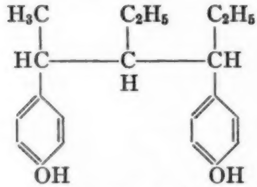
androsterone, etiocholan-3($\alpha$)-ol-17-one, and $\Delta^5$-androstene-3($\beta$), 17($\alpha$)-diol (6).

The partial synthesis of $\Delta^5$-androstene-3($\beta$), 16, 17-triol using dehydroisoandrosterone as the starting material has been achieved by Huffman & Lott (39). Turfitt (40) found that microbiological oxidation of steroid hydroxyl groups to keto-derivatives could be obtained with *Proactinomyces* spp.

An androgen with an oxygen atom at carbon 11 from the urine of a young woman suffering from virilism was isolated by Miller *et al.* (41) and appeared to be identical with the compound obtained by Mason (42, 43) from the urine of patients with disease of the adrenal cortex. A significant but considerably less amount of androstanediol-3($\alpha$),11($\beta$)-17-one was also isolated from the urine of normal men (44). This new ketosteroid is further shown to possess approximately one fourth as much activity as androsterone as measured by a chick comb test (41).

Furchgott *et al.* (45) have investigated the infra red absorption spectra of certain androgens and found that infra red spectroscopy may be used in the differentiaton of steroid isomers. Kumler (46, 47) continued his studies on the dipole moments of steroids and obtained the folowing values: androsteone, 3.70; $\beta$-androsterone, 2.95; $\Delta^5$-androstenediol-3($\beta$), 17($\alpha$), 2.89; $\Delta^5$-androstenediol-3($\beta$), 17($\beta$), 2.69; $\Delta^5$-androstenol-3($\beta$)-one-17, 2.46; testosterone, 4.32; *cis*-testosterone, 5.17; $\Delta^4$-androstenedione-3, 17, 3.32; androstanediol-3($\alpha$), 17($\alpha$), 2.29; androstanediol-3($\beta$), 17($\alpha$), 2.99; androstanedione-3, 17, 3.25; and 17-methyl-testosterone, 4.17. From these data, Kumler concluded that 3$\beta$- and 17$\beta$-hydroxyl groups do not have freedom of rotation and that either the 3$\alpha$ or 17$\alpha$ hydroxyl is restricted. Kumler's results also indicate that the A/B, B/C and C/D rings in these hormones are linked *trans*, each cyclohexane ring is in the chair form with the carbon atoms staggered.

*Estrogens*.—A number of 1,2,3-trialkyl derivatives of 1,3-di-(*p*-hydroxyphenyl) propane have been prepared and estrogenic activity determinations of these compounds indicate that one of the isomeric racemic forms of 2,4-di-(*p*-hydroxyphenyl)-3-ethylhexane (IV), which is called benzestrol or octofollin possesses a maximum activity in doses of 0.8 $\mu$g. (48). The physiological action of this synthetic compound was found to be identical in all respects



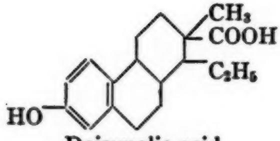
Benzestrol
IV

with that of natural estrogens and it is said to be relatively free from various toxic reactions (49, 50).

An alternative route to stilbestrol using *n*-butyric acid and *p*-bromoanisole as starting materials has been described (51). Wilds & Biggerstaff (52) have reported a synthesis of stilbestrol from $\alpha$-(*p*-methoxyphenyl)-*n*-butyric acid and anisole. A further recent synthesis is also reported by Rubin *et al.* (53) who used anisaldehyde cyanohydrin as starting material. Hudson & Walton (54) have described a new estrogenically active compound, 4:4′-dihydroxy-$\alpha$:$\beta$:2:2′-tetramethylstibene, as synthesized from *m*-methylanisole and 4-methoxy-2-methylbenzaldehyde. Solmssen (55) has given a comprehensive review on the chemistry of synthetic estrogens.

From the alkali degradation of estradiol, Miescher (56) isolated an active estrogen, 1-ethyl-2-methyl-7-hydroxy-1,2,3,4,9,10,11,12-octahydrophenanthryl-2-carboxylic acid, which is called doisynolic acid (V). The total synthesis of this estrogenic carboxylic acid has been achieved by Hunter & Hogg (57). The procedure involved a condensation of $\beta$-*m*-methoxyphenylethyl bromide with ethyl $\beta$-ketopimelate. When bioassayed by the Kahnt-Doisy method, using pure estrone as a standard, the product gave the typical estrus response in doses of 0.8 $\mu$g.

From an analogous degradation of equilenin and dihydroequilenin, Miescher (56) discovered another highly active estrogen, dehydrodoisynolic acid (VI). The total synthesis of this compound has been achieved by Heer *et al.* (58). Joel (59) found that dehydrodoisynolic acid is especially effective by mouth and it was claimed that it is the most effective estrogenic substance yet known. A synthesis of equilenin using 1-keto-7-methoxy-1,2,3,4-tetrahydro-




Doisynolic acid
V


Dehydrodoisynolic acid
VI

phenanthrene as the starting material, as previously employed by Bachmann *et al.* (60) has been described by Johnson *et al.* (61).

Infra red absorption spectra of various estrogens have been determined by Furchogott *et al.* (62); certain bands in the spectra due to the vibrations of atoms in the benzoid ring of the molecules may be used to differentiate the estrogens from those of other steroids. The fractionation of estrone, $\alpha$-estradiol and estriol in human urine was effected in a liquid chromatogram (63) and the estimation of each of these estrogens with the Kober reagent (64) has also been found to be satisfactory (65). A procedure for the estrogen assay using weanling guinea pigs as the test animal has been described by Hartman *et al.* (66). The dosage of the hormone is given in two hypodermic injections one on each side of the vulvar region; the disappearance of part of all of the vaginal closure membrane is taken as the endpoint. The test is highly sensitive, the threshold dose being $4 \times 10^{-7}$ mg. of the estrogen, and is particularly suitable for the estimation of the hormone content in the blood.

The nature of circulating estrogen has been investigated by Szego & Roberts (67, 68). It appeared that an equilibrium between estrogen and protein exists in the blood and more than 60 per cent of the hormone combines with the blood protein. Direct assay of the purified protein fractions of human plasma indicated that practically all of the protein-bound estrogen occurs in the lipoprotein fraction III-O of Cohn *et al.* (69). The hormone is found readily dissociated from the estrogen-protein complex during dialysis under simulated physiological conditions. Tracer technique has been employed to study the metabolism of a synthetic estrogen in mice (70). In the female animal, high concentration of radioactive $\alpha$-bromotriphenylethylene could be detected in the ovaries and uterus while the males were characterized by a selective fixation of the estrogen in the preputial glands.

The metabolism of estrogens has recently been reviewed by Marrian (71). An excellent assay on the biochemistry of steroid hormones has been presented by Pincus (72).

## INSULIN

*Chemical composition.*—The elementary analysis of insulin as given by Brand (73) is as follows: carbon 52.96 per cent, hydrogen 6.79 per cent, nitrogen 16.04 per cent, and sulfur 3.31 per cent. The value for sulfur is in good agreement with that reported by Miller & du Vigneaud (74) but is somewhat different from that given by Harrington & Scott (75) and Sahyun (76). Different results on nitrogen content have been reported. Chibnall *et al.* (77) found insulin to contain 15.54 per cent of nitrogen while Sahyun (76) gave 16 per cent, and Harrington & Scott (75) reported 14.49 per cent.

It is well known that crystalline preparations of insulin containing various metals can be obtained from ash-free material in the presence of zinc, nickel, cobalt and cadmium. Scott & Fisher (78, 79) found that such preparations may contain 0.52 per cent zinc, 0.41 per cent nickel, 0.77 per cent cadmium or 0.44 per cent cobalt. Variable amounts of zinc can be introduced into insulin molecules depending upon the conditions of crystallization. By the use of radioactive zinc, Cohn *et al.* (80, 81) were able to prepare crystalline insulin containing 0.31 to 0.65 per cent of zinc. Sahyun (76) has prepared a crystalline insulin containing 0.15 per cent of zinc.

Complete analyses of the amino acid content of insulin have been reported by Chibnall (82) and Brand (73), and their results are shown in Table I. The total nitrogen content in the hormone is practically accounted for by the amount of arginine, histidine, lysine, glutamic acid, apartic acid, cystine, tyrosine, alanine, valine, phanylalanine, serine, threonine, leucines, glycine, and proline. It may be noted that insulin is characterized by the high content of cystine, tyrosine, glutamic acid and leucines and by the absence of tryptophane and methionine. Brand (73) found that insulin contains 0.6 per cent cysteine, but other investigators (83,84,85) were unable to find the presence of this amino acid in the insulin molecule. Chibnall's values for basic amino acids were taken from the studies of MacPherson (86) and that for cystine was calculated from the sulfur value reported by Miller & du

Vigneaud 74). Values for arginine, lysine, and glutamic acid (87) as obtained from van Slyke nitrogen distribution method are in fair agreement with those listed in Table I.

If the data of Brand had been used and if the molecular weight of insulin were 44,600, the mean residue weight would be 114.6 and the number of peptide linkages per molecule of insulin would become 369. With the same assumption, the number of acid-binding groups per insulin molecule becomes 53 and that for base-binding

TABLE I

AMINO ACID CONTENT OF INSULIN*

| Constituent | Chibnall (82) | Brand (73) |
|---|---|---|
| Arginine | 3.00 | 3.50 |
| Histidine | 4.88 | 5.30 |
| Lysine | 2.44 | 2.60 |
| Glutamic Acid | 18.60 | 20.20 |
| Aspartic Acid | 5.68 | 6.8 |
| Amide-N ($NH_3$) | 1.68 | 2.15 |
| Cystine/2 | 12.50 | 11.0 |
| Tyrosine | 13.03 | 12.3 |
| Alanine | 4.60 | (2.9) |
| Valine | 7.49 | 8.8 |
| Phenylalanine | 8.09 | 7.9 |
| Serine | 5.18 | 5.8 |
| Threonine | 2.08 | 3.2 |
| Leucines | 15.68 | 16.3 |
| Glycine | 4.30 | 4.6 |
| Proline | 2.56 | 2.9 |
| Cysteine | | 0.6 |

* Figures in per cent of ash and moisture free protein.

groups 79. A similar conclusion for the number of acid and basic groups has been obtained from titration curves (88, 89, 90).

The number of peptide bonds in the insulin molecule has also been determined by Linderstrøm-Lang & Jacobsen (91) by titration of the increase in amino acid carboxyl groups after hydrolysis of the hormone. They found that the number of peptide bonds per nitrogen atom to be 0.753. On the assumption that the molecular weight of insulin was 44,600 with a nitrogen content of 16.04 per cent, the number of peptide bonds per molecule of insulin is

computed to be 384 which is somewhat higher than that derived from the analytical data of Brand.

As may be seen in Table I, Chibnall's analytical data are uniformly lower than that of Brand except for the value of tyrosine. From these data Chibnall concluded that the molecule of insulin is composed of either three or four submolecules and each submolecule has four peptide chains with a molecular weight of 12,000.

Sanger (85) has developed a new procedure for the identification of the terminal $\alpha$-amino groups in proteins and peptides using 2,4-dinitrofluorobenzene, and the method has been applied to insulin. It was shown that six free amino groups are present in a submolecule of the hormone; two of these are found in glycine residues, two in phenylalanine residues and two belong to the $\alpha$-amino groups of lysine. The free terminal phenylalanine residues have previously been demonstrated by Jensen & Evans (92). Thus, it is suggested that the insulin submolecule of molecular weight 12,000 contains four open peptide chains, two of these having terminal glycyl-residues and the other two phenylalanyl residues.

*Molecular weight.*—There is as yet no agreement on the molecular weight of insulin. The ultracentrifugal studies of Sjögren & Svedberg (93) gave a value of 35,100 by the equilibrium method. Polson (94) based on the diffusion constant, $D_{20,w} = 8.20 \times 10^{-7}$ sq. cm. per second, and sedimentation rate, $S_{20} = 3.47$ S, gave a somewhat higher value of 40,900. Miller & Andersson (95) redetermined the sedimentation and diffusion constants of insulin: $S_{20} = 3.55$ S and $D_{20,w} = 7.53 \times 10^{-7}$ sq. cm. per second; the molecular weight estimated from these data was found to be 46,000.

More recently, Gutfreund & Ogston (96) obtained a sedimentation constant of insulin as 3.34 S which is significantly lower than that reported by previous investigators. The value of Gutfreund and Ogston was the average of six determinations using two different crystalline preparations at concentrations of less than one per cent. If the diffusion constant of Miller and Andersson is employed, the molecular weight of insulin may be calculated to be 42,500; on the other hand, the computed molecular weight on the basis of Polson's diffusion rate becomes 39,000. It should be mentioned that these calculations are based upon the partial specific volume of insulin, $V = 0.749$, as determined by Sjögren & Svedberg (93).

From the analytical data on lysine, arginine, threonine and histidine, Brand (73) calculated the molecular weight of insulin to be 44,600. Chibnall assumed the insulin molecule to consist of either three or four submolecules each with a molecular weight of 12,000, i.e., the molecular weight of insulin might be 36,000 or 48,000. Based upon the metal content of insulin crystals, Scott (97) deduced a value of 40,000 for the molecular weight of the hormone. If one accepts Sahyun's zinc value (76) as the lowest value for zinc-insulin, and the insulin molecule contained one atom of zinc, the minimum molecular weight will be 43,500. From x-ray data on crystalline zinc-insulin, Crowfoot (98) computed a molecular weight of about 36,000. Table II summarizes the values given in the literature for the molecular weight of insulin obtained by different methods.

TABLE II

Molecular Weight of Insulin

| | |
|---|---|
| Ultracentrifuge: | 35,100 (93) |
| | 40,900 (94) |
| | 46,000 (95) |
| X-ray: | 36,000 (98) |
| Minimum by Analysis: | 44,600 (73) |
| | 48,000 or 36,000 (82) |

*Solubility.*—The difference in amino acid content of two crystalline insulin preparations obtained by two laboratories as illustrated in Table I may be taken as an indication that the hormone is not a single pure protein. Electrophoresis (99), diffusion (95), and ultracentrifuge (93, 95) studies have shown that crystalline insulin behaves as a homogeneous substance. Lens (100) applied the solubility method to examine the purity of insulin and found that solubility curves of insulin samples obtained at pH 4.95 and pH 6.0 are of the solid solution type. It would be desirable to investigate solubility behavior of insulin in other solvents; the solubility curves might become the mixture type so that a procedure may be worked out to obtain an absolutely pure preparation of crystalline insulin.

The solubility of insulin in water has been determined by Cohn

*et al.* (80, 81) who found that the solubility of the zinc-insulin in water at 5° was of the order of 0.01 gm. per liter and that the solubility was increased in the presence of glycine.

Table III presents a summary of physicochemical characteristics of insulin.

TABLE III

PHYSICOCHEMICAL CHARACTERISTICS OF INSULIN

| | |
|---|---|
| Diffusion Constant, $D_{20}\times10^{7}$ | 8.20 (94) |
| | 7.53 (95) |
| Sedimentation Constant, $S_{20}\times10^{13}$ | 3.47 (93) |
| | 3.55 (95) |
| Isoelectric Point, pH | 5.30–5.35* |
| | 5.4** |
| Partial Specific Volume, V | 0.749 (93) |
| Dissymmetry Constant, $f/f_0$ | 1.18 (95) |
| Solubility: water at 5°, gm. per liter | 0.01 (81) |
| 1.0 *M* glycine at 5°, gm. per liter | 0.069 (81) |
| Relaxation Time in propylene glycol solution in seconds at 25° | $1.7\times10^{-8}$ (80) |
| Refractive Index Increment at 436$\mu$ | $1.88\times10^{-3}$ (113) |

* Wintersteiner, O., and Abramson, H. A., *J. Bill. Chem.*, **99**, 741 (1933).
** Howitt, F. O., and Prideaux, E. B. R., *Proc. Roy. Soc.*, [B] **112**, 13 (1932).

*Stability.*—Sjögren & Svedberg (93) have found the stability region of insulin to be between pH 4.5 and pH 7.0; the insulin molecule dissociates into products of low molecular weight outside this range as demonstrated by the rapid fall in the sedimentation rate. If the pH of the solution is not too far removed from the stability region, the changes are reversible both on the acid and the alkaline side. Harrington & Neuberger (89) also observed these reversible changes from titration studies. Miller & Andersson (95) showed that at pH 8.5 the sedimentation constant decreased considerably and the molecule dissociated continuously into smaller particles. Recent studies of Gutfreund & Ogston (96), cannot confirm these findings and they found the normal sedimentation constant was still obtained at pH 9.05. At pH 3.4, however, the sedimentation constant was significantly lower and the insulin became polydisperse. It is of interest to note that the biological activity of insulin does not decrease appreciably (97) after being heated at 52° in a solution of pH 2.5 and that the heat

precipitate of insulin in acid exhibits 80 per cent of the original biological potency after it was dissolved in dilute alkali and immediately acidified (101).

*Modifications.*—Langmuir & Waugh (102) have shown that when a 2 per cent insulin hydrochloride solution at pH 2.0 was heated at 100° in a water-bath for thirty minutes, it formed a stable birefringent thixotropic gel indicating that the protein molecule has been modified to give anisodiametric micelles. Electron micrographs of this material (103) indicate that insulin fibrils have a length of several microns with uniform widths of approximately 200 Å. A dispersion of these fibrils in alkali solutions loses its characteristic double refraction and viscosity; the solution thus produced resembles that of native insulin, and it can be converted again to produce a thixotropic gel showing static double refraction (103). It has also been demonstrated (104) that fibril formation appears to be a prerequisite for the formation of floccules and that the floccules are spherites. Waugh (104) found that the rate of spherite formation from fibrils increases with increasing hydrogen ion concentration, protein concentration, neutral salt concentration, temperature and fluidity. It is remarkable that native insulin can be converted into fibrous form (105) when a native insulin solution is mixed with some insulin fibrils.

Rothen *et al.* (106) obtained completely unfolded insulin films of 7 to 9 Å in thickness[3] at pH 5.6 and showed that these insulin films still possess the original biological potency. This suggests that some small structural units within the molecule are responsible for the hormonal action. Bischoff (108) found the activity of insulin unimpaired by denaturation with sodium lauryl sulfate. It has already been shown by Miller & Andersson (109) that insulin dissociates into subunits in the detergent solutions and that an insulin detergent complex with a micellar weight of about 27,600 is formed.

The reactions of insulin with iodine (89), ketene (110) and reducing reagents (111, 112, 113) established the fact that tyrosine[4]

[3] The effect of pH on the thickness of insulin film has been studied by Clowes (107).

[4] Crammer & Neuberger (114) determined the state of tyrosine groups in insulin by spectrophotometric titration and found that the pK of the phenolic group increases as the pH is raised. This change is said to be due to the increasing net charge of the protein.

and disulfide groups are essential for the hypoglycemic action of the hormone. Reitz *et al.* (115) have prepared acid sulfate esters of insulin by treatment with concentrated sulfuric acid at low temperatures and found that nearly all of the aliphatic hydroxyl groups were esterified. Biological assay of these insulin sulfates (116) showed that no loss of the hypoglycemic activity was observed. These results indicate that either the aliphatic hydroxyl groups or the net charge do not participate in the biological action of insulin.

Jensen (117), Soskin & Levene (118), and Best (119) have reviewed the properties of insulin.

## Anterior Pituitary

It is well established that there are at least six hormones identifiable as individual substances in extracts of anterior pituitary tissue—namely, follicle-stimulating (FSH), interstitial-cell stimulating (luteinizing, ICSH), lactogenic (prolactin), thyrotropic, adrenocorticotropic (ACTH) and growth hormones. Of these hormones, four have already been prepared in chemically pure form. In addition, the claim has been made that the follicle-stimulating hormone has been obtained in "biologically pure" state (120) and this hormone appears to be chemically distinct from all others. Thyrotropic hormone has been highly purified but not yet isolated (121).

One of the most interesting facts to come from physicochemical studies of the four pure hormones has been the demonstration that the hormones isolated from glands of different mammalian species are not identical substances. For instance, ox prolactin has a higher tyrosine content than that of the sheep hormone (122). Other remarkable differences were observed in the luteinizing hormone isolated from sheep (123) and from swine pituitaries (124). On the other hand, the adrenocorticotropic hormone obtained either from sheep (125, 126) or from swine (127) glands appears to be identical although porcine glands have a higher hormonal content. In Table IV, a summary of certain physicochemical characteristics of the four isolated hormones is presented.

*Follicle-stimulating hormone* (*FSH*).—The sheep and swine pituitaries are rich in the follicle-stimulating substance and therefore they are commonly used to obtain a potent FSH preparation. The extraction of pituitary tissue (fresh or acetone dried material)

is made either with saline or alcoholic solutions. Chemically the follicle stimulating hormone is in one respect unique in that it is the only known anterior hypophyseal hormone soluble in half-saturated ammonium sulfate. Highly purified FSH possesses a high carbohydrate content (128, 129). In addition, the hormone is resistant to tryptic digestion when a crude enzyme preparation is employed (130, 131). There are methods which enable one to obtain a so-called "biologically pure" follicle-stimulating hormone

TABLE IV

Physicochemical Properties of ICSH, ACTH, Lactogenic and Growth Hormones

| Determination | ICSH | | ACTH | | Lactogenic Hormone | | Growth Hormone (Ox) |
|---|---|---|---|---|---|---|---|
| | Sheep | Swine | Sheep | Swine | Sheep | Ox | |
| N, % | 14.2 | 14.93 | 15.65 | 15.47 | 15.86 | 16.50 | 15.65 |
| S, % | | | 2.3 | 2.33 | 1.79 | 2.0 | 1.3 |
| Cystine, % | | | 7.19 | | 3.11 | 3.4 | 2.25 |
| Methionine, % | | | 1.93 | | 4.31 | | 3.06 |
| Tryptophane, % | 1.0 | 3.8 | | | 1.25 | 1.3 | 0.84 |
| Tyrosine, % | 4.5 | | | | 4.53 | 5.7 | 4.3 |
| Molecular Weight, *M* | | | | | | | |
| Osmotic Pressure | 40,000 | | | | 26,500 | 26,500 | 44,250 |
| Sedimentation | | 100,000 | 20,000 | 20,000 | | 32,000 | |
| Diffusion constant, $D_{20} \times 10^7$ | | | 10.4 | | 9.0 | 7.5 | 7.15 |
| Sedimentation constant as Svedberg units, S | 3.6 | 5.4 | 2.08 | 2.04–2.11 | | 2.65 | |
| Isoelectric Point, pH | 4.6 | 7.45 | 4.65–4.70 | 4.70–4.80 | 5.73 | 5.73 | 6.85 |
| Partial specific volume, $V_1$ | | | | | 0.721 | | 0.760 |
| Relative viscosity | | | | | 6.65 | | 7.64 |
| Dissymmetry constant, $f/f_0$ | | | 1.1 | | 1.29 | | 1.31 |

preparation, i.e., a preparation free from other active contaminants. The procedure of Greep *et al.* (120) is based on the fact that FSH is soluble in a pH 4.4 acetate buffer containing 20.5 per cent sodium sulfate whereas the luteinizing component is insoluble in this solvent, and the product is shown to be inhomogeneous in ultracentrifugal and electrophoretic experiments (132). McShan & Meyer (133, 134) employ the tryptic digestion technique to obtain a biologically pure FSH.

The preparation as described by Fraenkel-Conrat *et al.* (135) contains 13.1 per cent nitrogen. It is generally agreed that FSH proteins are very soluble in water; in the absence of electrolytes,

they are soluble in 50 per cent acetone, 70 per cent alcohol (135) and 50 per cent dioxane (136). The follicle stimulating potency is comparatively stable; in solutions of pH 7 to 8 the activity is retained at 75° C. for thirty minutes (133) but it is destroyed at 60° C. for fifteen minutes in 50 per cent alcoholic solution (136). Chow (132) has estimated the isoelectric point of his FSH preparation from swine glands to be about pH 4.8.

*Thyrotropic hormone.*—The first attempts to concentrate the thyrotropic principle from the pituitary extract were carried out by Loeb & Bassett (137) and Janssen & Loeser (138). The hormone has not yet been isolated in pure form, although highly purified preparations have been reported (121).

Both acid (139, 140, 141) and alkaline (142 to 145) extracts of pituitary tissue have been used for the preparation of the thyrotropic substance; saline extraction (138, 146) is also applicable. To concentrate the thyrotropic hormone from these extracts many fractionation techniques have been tried, such as alcohol or acetone (139, 140), salt (139, 145) or protein precipitants (121, 141, 142, 147, 148). It is of great interest to note that the thyrotropic principle is soluble in the solution of some protein precipitating agents, namely, trichloroacetic acid, sulfosalicylic acid, lead acetate etc. This indicates that the hormone may possess a rather low molecular weight and would harmonize with the fact that ultracentrifugation does not concentrate the thyroid-stimulating hormone (149, 150).

Adsorption methods have also been employed in an effort to purify the thyrotropic hormone. Jorgensen & Wade (148) found that the hormone is adsorbed by permutit at pH 4.5 and subsequently concentrated by precipitation with uranium acetate. Repetition of these procedures does not appreciably further concentrate the hormone. Ciereszko (121) employed lead acetate and trichloroacetic acid to remove the contaminating proteins and obtained a highly purified thyrotropic preparation; a total dose of 0.001 mg. of the material causes histological changes in chick thyroids when injected over a period of five days.

The purified thyrotropic preparation contains 1.2 per cent sulfur and has no phosphorus (121). Fraenkel-Conrat *et al.* (139) found hexose and glucosamine in their preparation. The hormone is highly soluble in water (121, 139). White (151) estimated the molecular weight of the hormone as approximately 10,000 from ultracentrifugal data. The thyrotropic activity is destroyed by

ketene and cysteine treatments (139). Chow *et al.* (131) found that the thyroid stimulating action is inactivated by crystalline preparations of chymotrypsin, trypsin and pepsin but not by digestion with papain.

*Luteinizing hormone* (*ICSH*).—A method for isolation of swine ICSH in pure form has been described by Shedlovsky *et al.* (124) and that for the sheep hormone by Li *et al.* (123). Fevold *et al.* (145) have also reported a method for the preparation of a highly purified sheep ICSH but their product contains two components in electrophoretic and ultracentrifugal studies.

It may be noted from Table IV that both molecular weight and isoelectric point of the sheep luteinizing hormone differ widely from the swine protein. Both sheep and swine ICSH contain carbohydrate but the content differs significantly (128, 152). The tryptophane content of the two hormones has also been found to be different (152). The fact that the interstitial cell stimulating hormones isolated from swine and sheep glands are chemically different entities is further demonstrated by immunological reactions (153).

The reactions of the hormone with ketene and cysteine cause a decrease of the interstitial cell stimulating potency (123, 154, 155). The enzymic digestions with crystalline chymotrypsin and trypsin have also been shown to destroy the hormonal action (131).

*Lactogenic hormone* (*prolactin*).—A highly purified and potent lactogenic hormone preparation was first described by Lyons (156, 157) in 1937. In the same year, White *et al.* (158) reported the preparation of a crystalline protein possessing crop-stimulating activity; in the preliminary note, there was, however, no data concerning the biological and chemical purity of the crystalline preparation. It was not until 1942 that White *et al.* (159) published a satisfactory identification of the crystalline protein with the hormone. In the meantime (1940–1941) Li *et al.* (160 to 163) showed that their lactogenic hormone preparation behaved like a pure protein as judged by electrophoretic and solubility studies.

The hormone isolated from either ox or sheep pituitaries shows no difference in crop-sac stimulating potency. Bischoff & Lyons (164) were unable to differentiate the ox and sheep hormones through the use of precipitin or anaphylaxis reactions. It was further found that they can not be distinguished in electrophoresis experiments (162). The two hormones have the same

molecular weight and content of tryptophane, arginine, cystine, and methionine (163, 165). However, the hormone isolated from ox pituitaries can easily be differentiated by their tyrosine content and solubility behavior. For instance, the sheep hormone is more soluble in acidic sodium chloride solutions than the ox preparation (161, 163), while the ox hormone is more soluble than the sheep in alcoholic solution (166). As already mentioned, the tyrosine content of ox lactogenic hormone is consistently higher than that of the sheep; ox protein has 5.73 per cent tyrosine whereas that from sheep contains 4.53 per cent (163, 167). Analyses for other amino acids may reveal further differences in the hormone prepared from these two species.

The hormone has no cysteine or sulfhydryl groups as shown by the nitroprusside, phosphotungstate or iodine test (168, 169). Both Sullivan and phosphotungstate methods give a cystine content of 3.0 per cent in prolactin (169); a somewhat higher value (3.36 per cent) has been reported (159). Li (165) found 3.11 per cent cystine in the hormone using the method of Baernstein. Since both the method of preparing prolactin and methods of analysis for cystine were not the same, the cystine values which have been reported should be regarded as in satisfactory agreement. If we take 4.31 and 3.11 per cent as the methionine and cystine content respectively, the total sulfur in the hormone is accounted for within the limits of error (165).

The tryptophane content of lactogenic hormone seems to vary with the method of analysis. The nitrous acid method of Lugg and the method of Folin and Marenzi give a value of 1.3 per cent (159, 168), but a higher value (2.5 per cent) is obtained by the glyoxalic acid procedure (163). Using the *p*-dimethylaminobenzaldehyde method as modified by Sullivan *et al.*, the tryptophane content of the hormone is found even higher, 3.1 per cent (170). By the method of Horn & Jones (171), we have obtained a value of 1.27 per cent (170). Recent determinations with a microbiological method show that the hormone contains 1.23 per cent tryptophane (172). From these data it may be said that the most probable value for the content of tryptophane in lactogenic hormone is 1.25 per cent. Higher values obtained by colorimetric methods may be due to certain characteristics of the tryptophane groups in the hormone molecule.

Iodine reacts exclusively with the tyrosine groups in lactogenic

hormone and iodinated hormone has no lactogenic potency (168). Cysteine and thioglycolic acid inactivate the hormone under certain conditions (173). It has been shown that the reaction of the hormone with ketene caused a destruction of the biological activity and that the decrease in crop stimulating potency is due to coverage of the amino groups (174, 175). The employment of other reagents, such as phenyl isocyanate (177) and nitrous acid (176) have also demonstrated the essentiality of the amino groups for the specific biological effects of the hormone. The esterification of carboxyl radicals in the hormone with methyl alcohol has been shown to cause a loss of lactogenic activity (178). The hormone is also inactivated by the detergents (179). There are thus far no indications of the existence of a prosthetic group or groups in the lactogenic hormone molecule; the results summarized above suggest rather that the structural make-up of the whole molecule is necessary for its physiological role.

*Adrenocorticotropic hormone* (*ACTH*).—The first evidence for the occurrence of an adrenocorticotropic substance in pituitary extracts was given by Smith (180), and it appears that although hypophysectomy decreases the adrenal cortex it is without effect on the medulla. The isolation of the adrenocorticotropic hormone has been achieved independently by two laboratories (125, 126, 127). Li *et al.* (125, 126) employed sheep glands as their starting material whereas Sayers *et al.* (127) prepared their hormone from swine pituitaries. The hormones isolated from these two species appeared apparently identical with respect to the molecular weight, isoelectric point and biological potency. The hormone contains no carbohydrate, phosphorus or cysteine but it has a high content of cystine (7.19 per cent). The methionine and cystine content account fully for the amount of sulfur in the protein (181).

In neutral solutions, adrenocorticotropic hormone is remarkably resistant to heat treatment (126). The reactions of the hormone with ketene, nitrous acid, formaldehyde and iodine have been studied (182) and the results suggest that both the free amino and tyrosine groups are essential for activity. The hormone remained active after certain pepsin digestions but was destroyed by the enzymic action of trypsin (126).

Recent experiments (183) have shown that the nonprotein nitrogen, as judged by the trichloroacetic acid precipitation, of the peptic hydrolysate of adrenocorticotropic hormone contains the

hormonal activity. The adrenocorticotropically active non-protein nitrogen, which dialyzes through collodion membranes, can also be demonstrated in hydrochloric acid hydrolysates. The average number of amino acid residues of the peptides present in these hydrolysates has been determined and found to be about seven.

Adrenocorticotropic hormone exhibits a retardation of the somatic growth of normal rats (184, 185). When adrenocorticotropic hormone is injected simultaneously with the growth hormone in hypophysectomized rats a counteraction exists between these two substances (186, 187). The fact that the adrenocorticotropic hormone may be regarded as a specific growth inhibiting substance is further indicated by its effect on the increase of urinary nitrogen excretion in normal rats (188). It has also been shown that adenocorticotropic hormone reduces the alkaline phosphatase content in the plasma of both hypophysectomized and normal rats (189) and that the effect is neutralized by growth hormone injections (170).

The production of glycosuria in normal rats with adrenocorticotropic hormone has been demonstrated by Ingle *et al.* (190). Similar results were obtained with 17-hydroxycorticosterone (191). The experiments indicate that the adrenal cortex after stimulation with adrenocorticotropic hormone increases its production and secretion of the steroids[5] which affect carbohydrate metabolism. It is not yet clear as to whether electrolyte metabolism is also under the control of the adrenocorticotropic hormone.

The diabetogenic action of adrenocorticotropic hormone has furthermore been demonstrated in alloxan induced diabetic rats (192); the results clearly show that the hormone enhances the diabetes and opposes insulin in its effects on nitrogen and glucose excretion. It has also been shown that adrenocorticotropic hormone exercises a beneficial effect on the resistance of normal rats to low atmospheric pressure (193) and that the hormone greatly increases the work performance of hypophysectomized rats (194).

Evidence has been advanced by Dougherty & White (195, 196) that the rise in serum proteins following adrenocorticotropic hormone administration or by the injection of adrenal cortical

[5] Unpublished experiments which were carried out in collaboration with Drs. H. L. Mason and E. H. Ryerson of the Mayo Clinic have shown that the administration of adrenocorticotropic hormone to a human subject causes an increase in the excretion of cortin-like substances, 17-ketosteroids and nitrogen.

hormones is the result of elevation of the antibody-containing serum globulins but this has not received confirmation. The prior work of Levin & Leathem (197), confirmed in this laboratory (198), demonstrated that adrenal cortical extracts or adrenal cortical stimulation by adrenocorticotropic hormone in the rat is not attended by a rise in serum globulin. There is, in fact, a definite rise in serum albumin.

Sayers *et al.* (199, 200, 201) reported that the injection of adrenocorticotropic hormone into rats and guinea pigs produced a prompt fall in adrenal cholesterol and ascorbic acid. The effect of adrenocorticotropic hormone on adrenal cholesterol was also observed by Carreyett *et al.* (202). The change of ascorbic acid in the adrenals following the administration of adrenocorticotropic hormone has been used as a basis for the assay of the hormone (203, 204).

The lack of renotropic effect of adrenocorticotropic hormone has been demonstrated by two laboratories (205, 206). The nephrosclerotic activity of anterior pituitary is not due to the adrenocorticotropic hormone but it has been shown to be caused by an unknown pituitary factor (207). This unknown factor probably stimulates the adrenal cortex to enhance the secretion of desoxycorticosterone-like compounds (208, 209). The role of the adrenals in the production of the general adaptation syndrome and the diseases of adaptation has been discussed by Selye (210).

Besides pituitary tissues, there are indications that the adrenocorticotropic hormone may be found in the serum of pregnant mares (211) and in female human urine (212). The adrenocorticotropic substance in normal female urine is found to be nondialyzable and thermolabile (213). On the other hand, Reiss *et al.* (214) have found that the adrenocorticotropic material in the urine from pregnant women is thermostable.

*Growth hormone.*—The viscosity coefficient, diffusion constant and partial specific volume of the growth hormone have been determined (215); from these data the dissymmetry constant of the protein is computed to be 1.31 indicating that the molecule is not spherical in shape. It was also found that the hormone is denatured in pH 4.0 acetate buffer of ionic strength 0.10 as judged by the increment in the intrinsic viscosity and molecular weight, but its growth-promoting action is essentially unchanged.

The sulfur content (1.3 per cent) in the hormone is accounted for by 3.06 per cent methionine and 2.25 per cent cystine (181, 215). When the growth hormone was treated with nitrous acid or ketene, the product exhibited no biological activity. It was concluded from these experiments that free amino groups are essential for the growth promoting action (183).

The content of arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine in the growth hormone protein has been determined with the microbiological technique (216). In Table V, the amino acid composition of the growth hormone is summarized; values for tyrosine and cystine (181, 217) are ob-

TABLE V

COMPOSITION OF GROWTH HORMONE
(N content of protein = 15.65%)

| Constituent | N as % Protein N | Amount per 100 gm. protein | Minimal Mol. Wt. | Assumed number of residues | Calculated Mol. Wt. |
|---|---|---|---|---|---|
| Amide-N (Ammonia) | 6.3 | 1.2 | 1420 | 30 | (42,600)[a] |
| Arginine | 18.8 | 9.1 | 1910 | 25 | 47,700 |
| Aspartic Acid | 6.1 | 9.0 | 1480 | 32 | 47,300 |
| Cystine | 1.7 | 2.25 | 10700 | 4 | (42,808)[a] |
| Glutamic Acid | 7.9 | 13.0 | 1130 | 42 | 47,400 |
| Glycine | 4.5 | 3.8 | 1980 | 24 | 47,400 |
| Histidine | 4.6 | 2.65 | 5850 | 8 | 46,800 |
| Isoleucine | 2.7 | 4.0 | 3280 | 14 | 46,000 |
| Leucine | 8.3 | 12.1 | 1080 | 44 | 47,700 |
| Lysine | 8.7 | 7.1 | 2060 | 23 | 47,400 |
| Methionine | 1.7 | 2.9 | 5140 | 9 | 46,200 |
| Phenylalanine | 4.3 | 7.9 | 2090 | 23 | 48,000 |
| Threonine | 6.8 | 9.0 | 1320 | 36 | 47,600 |
| Tryptophane | 0.7 | 0.84 | 24500 | 2 | 48,700 |
| Tyrosine | 2.1 | 4.3 | 4220 | 11 | 46,400 |
| Valine | 3.0 | 3.9 | 3000 | 16 | 48,000 |
| Total found | 88.2 | 93.04 | | 343 | |
| Mean ± Standard deviation | | | | | 47,300 ± 600 |

[a] Values in bracket are omitted from the mean.

tained with colorimetric methods. It may be noted that the tryptophane content as estimated by the nitrous acid method of Lugg is somewhat higher (217) than that determined by the microbiological procedure, while values for glutamic acid and methionine are in good agreement with results reported previously (181, 218). There are no data given for alanine, proline and serine. The summation of the known amino acid nitrogen together with the amide nitrogen accounts for 88.2 per cent of the protein nitrogen. An inspection of the data given in Table V reveals no peculiarity; the amount of each amino acid in the hormone is within the normal values found in other proteins. The calculated data listed in the last three columns show that the molecular weight of the protein is about $47{,}300 \pm 600$. This value is in fair agreement with that deduced from osmotic pressure measurements (217).

The free amino nitrogen of the hormone (217) as determined in the manometric Van Slyke apparatus was found to be 0.76 per cent indicating that the hormone has 25 moles of free amino nitrogen per mole of protein (47,300). As shown in Table V, the growth hormone contains 23 moles of lysine. Thus, it may be suggested that the protein consists of at least 340 amino acids arranged in two subunits or chains.

There has been some discussions as to the ability of the growth hormone to cause continuous growth in normal or in hypophysectomized rats (219). Since practically all experiments reported have been carried out with only partially purified growth-promoting extracts, it is naturally difficult to decide whether the results were due to the growth hormone itself or to other contaminating proteins. The availability of the isolated hormone provides an opportunity to investigate this important problem. Results with normal adult female rats after the administration of the hormone for 435 indicate that growth continued during the whole period with no sign of a lack of responsiveness (220). The average gain of the 8 experimental rats was 293 gm., that of the controls 57 gm. The average body length at autopsy of the experimental rats was 45.5 cm., of the controls 40.9 cm. Liver, kidneys, heart, stomach and intestine increased in weight in proportion to body weight. The endocrine organs were not increased proportionally to body weight, as might be anticipated from the absence of specific hormonal stimulants in the preparations. A similar experiment (unpublished)

with hypophysectomized female rats also shows that the growth hormone induces continuous growth in such animals and there is no indication of refractoriness with over four hundred days of injection.

Since true growth is generally interpretated as the accumulation of proteins, it is anticipated that an important function of the growth hormone is to retain nitrogen. Recent studies with normal rats (221) indicate that injections of growth hormone induce a significant lowering of the urinary nitrogen within twenty-four hours. Alloxan diabetic rats also retain nitrogen after growth hormone treatment (192). The nitrogen retaining effect of the growth hormone has also been demonstrated in rats with bilateral fracture of the femur (222).

Another characteristic function of the growth hormone is to bring about a specific stimulation of the epiphyseal cartilages in hypophysectomized rats. Becks *et al.* (223) have shown that administration of growth hormone to hypophysectomized rats, even after postoperative intervals of a year or longer was able to reawaken chrondogenic and oesteogenic processes in the epiphyseal cartilage of the tibia to an extent comparable to that seen in normal, young, growing rats.

The growth hormone was found to increase the alkaline phosphatase content in the plasma of hypophysectomized rats (170). It was also shown that the hormone causes a rise in the plasma inorganic phosphorus level of such animals (224). Reifenstein *et al.* (225) have proposed the use of the serum phosphorus level as an index of growth hormone activity.

The preparation and chemistry of anterior pituitary hormones have been summarized by White (226). Li & Evans (227) have reviewed the properties of growth and adrenocorticotropic hormones.

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# FAT-SOLUBLE VITAMINS[1]

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## VITAMIN A

### Chemistry

$\beta$-carotene, which at first seemed so readily purifiable by crystallization and chromatography, has been unusually embarrassing to those who have certified that this or that preparation was "pure," only to have a more potent preparation described shortly afterwards. The optical properties of the latest crystals, which may well be near the ultimate in purity, are described below in a table taken from the article describing their preparation by Devine *et al.* (1).

TABLE I

New Preparation of $\beta$-Carotene (1)

| Solvent | Wavelength of Main Band (m$\mu$) | E(1%,1cm.) | Previous Literature |
|---|---|---|---|
| Cyclohexane | 456 | 2490 | 2400 (456 m$\mu$) (2) |
| Chloroform | 465 | 2370 | 2200 (463 m$\mu$) (3)<br>2310 (463 m$\mu$) (4) |
| Hexane | 453 | 2610 | 2580 (450 m$\mu$) (5)<br>2540 (450 m$\mu$ in petrol) (6)<br>2500 (450 m$\mu$ in petrol) (3) |
| Benzene | 465 | 2370 | — |

Another crystalline form of vitamin A different from the form first prepared from fish liver oils by Robeson & Baxter has been discovered by these chemists (7, 8). The better known form which they had previously crystallized and made derivatives of has a melting point of $63-64°$ and an absorption maximum at 325 m$\mu$ with a value of 1750 for E(1%,1cm.). The newly discovered sub-

[1] This review covers the period from October, 1945 to December, 1946.

* Communication No. 110 from the laboratories of Distillation Products, Inc., Rochester, New York.

stance which they have called neovitamin A melts at 59 – 60° and has an absorption maximum at 328 mμ with a value of 1675 for E(1%,1cm.). The two forms seem to be geometrical isomers and each can be converted into mixtures of the two by iodine catalysis of the anthraquinonecarboxylic ester. The two forms have about the same biological activity and are converted biologically into mixtures. A mixture of the two can be analyzed by following the rate of reaction with maleic anhydride; neovitamin A forms a derivative much more slowly. By such studies it has been shown that most liver oils contain vitamin A and neovitamin A in approximately a 2:1 mixture.

The methyl ether of vitamin A has been prepared by Cawley (9, 10) and by Hanze *et al.* (11). The light yellow crystals, melting point 33–34°C., had the full biological activity of vitamin A alcohol, as well as the same ultraviolet absorption curve.

Vitamin A aldehyde has been prepared by the oxidation of vitamin A by Hawkins & Hunter (12) and by the oxidation of β-carotene by Hunter & Williams (13). The aldehyde could be reduced with aluminum isopropylate to give vitamin A.

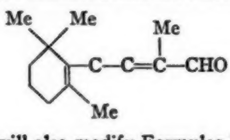
## Synthesis

The most interesting recent developments in vitamin A chemistry have, of course, been the recent syntheses of compounds which possess vitamin A activity. Milas (14, 15) has made compounds with structures corresponding to the ethers and esters of vitamin A by several different methods. The formulas[2] for the synthesis labelled "Milas A" show one of the typical procedures that is followed (16).

β-ionone (I) was reacted with ethyl chloroacetate and the product saponified to give an aldehyde[3] (II) which on further reaction

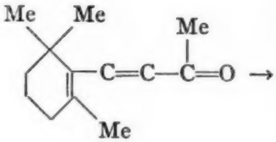
[2] To simplify the formulas the hydrogen atoms have been omitted from the carbon linkages.

[3] Heilbron *et al.* (17) have indicated that the correct structure for II is:

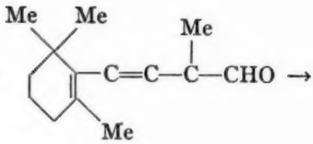



(This change in structure will also modify Formulas III, IV and IX.) This same paper also proposed the general type of syntheses described as "Milas A" and "Milas B."

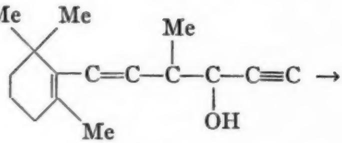
*"Milas A"*



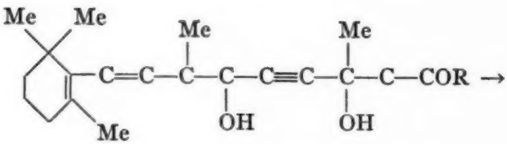
β-ionone I



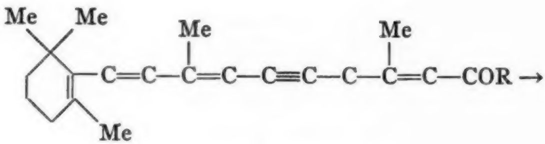
II



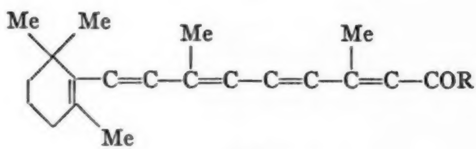
III



IV



V

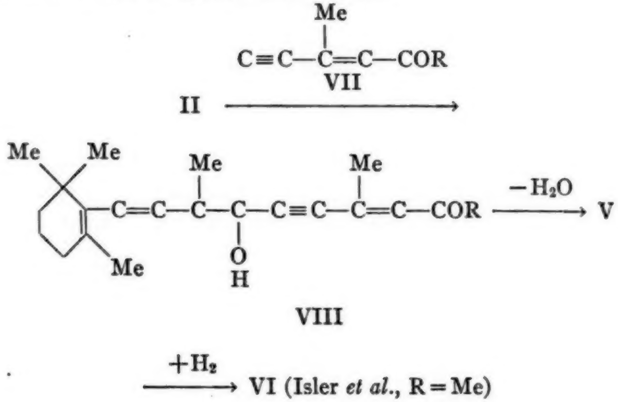


VI

Vitamin A ethers and esters

with lithium acetylide in liquid ammonia gave the acetylene carbinol (III). This was condensed, via the Grignard reaction, with a ketone, $CH_3COCH_2CH_2OR$, in which R could be either an alkyl or acyl group. The glycol so formed (IV) was dehydrated to give an acetylenic compound (V) which upon hydrogenation with a poisoned catalyst gave the vitamin A ethers or esters represented by Formula VI. An alternate synthesis, "Milas B," (16) in-

*"Milas B"; Isler, Kofler, Huber & Ronco*

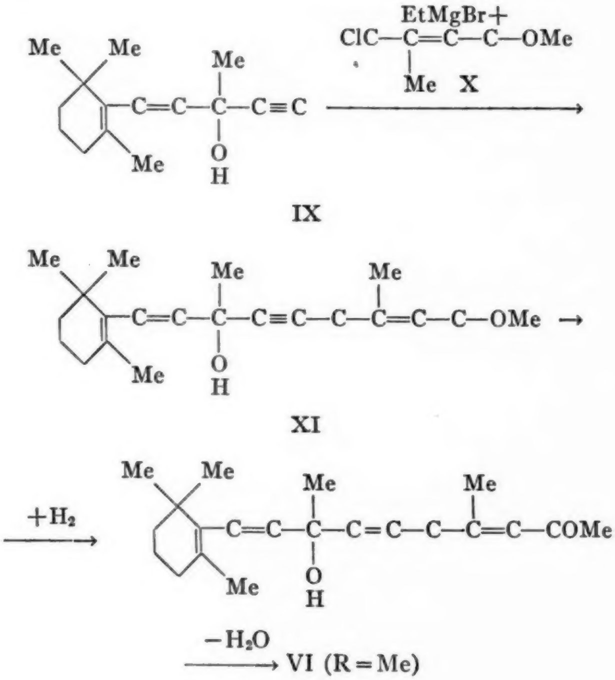



volves the reaction of the aldehyde II with the pentenyne compound (VII) to give a hydroxy acetylene derivative (VIII) which on dehydration and hydrogenation will give the corresponding vitamin A derivative (VI). While these compounds prepared by Milas seem to have the proper chemical composition and reaction with antimony trichloride, as well as almost exactly the same absorption spectrum as natural vitamin A substances, the biological activity was much lower. Most of the assays showed that the synthetic vitamin A derivatives had potencies of the order of 50,000 to 100,000 U.S.P. units per gm. instead of the potency of natural vitamin A, 3.5 million units per gm.

Isler *et al.* (18) carried out reactions very much like the "Milas B" synthesis. They prepared in this way a compound with a structure corresponding to the methyl ether of vitamin A, and they report that its biological activity is at least as high as that

of $\beta$-carotene. The anomalous biological activities of his materials prompted Milas to suggest that the synthetic products are mixtures of stereoisomers of the *cis-* and *trans*-type. Apparently Milas' preparation did not contain stereoisomers like ordinary vitamin A or the equally active neovitamin A of Baxter & Robeson (8).

*Oroshnik*




XII

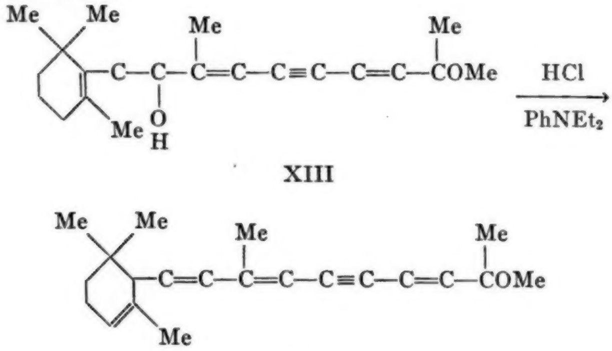
The structure of vitamin A, of course, indicates that more isomers than these two could possibly exist.

A preliminary report by Oroshnik (19) describes the synthesis of a substance with the structure of vitamin A methyl ether. While at first his reactions seem similar to those of Milas and of Isler *et al.*, they are quite different in that Oroshnik starts

with the acetylene group connected directly with the $\beta$-ionone structure. The building up of the rest of the chain is somewhat similar to the other syntheses, but the products are different in that the acetylene group is one carbon nearer the $\beta$-ionone ring. At the final dehydration step an allylic rearrangement takes place to give the desired product. While the preliminary report of Oroshnik mentions that the absorption maximum of this product is at 315 m$\mu$ instead of at 325 m$\mu$ ordinarily expected for such an ether, a private communication from him states that the distortion in the shape of the absorption spectrum was found to be due, in part at least, to a compound with four conjugated double bonds and that his product actually has biological activity.

Cymerman *et al.* (20), whose laboratory has studied many

*Cymerman et al.*



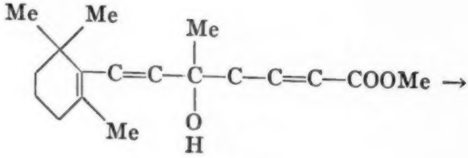

XIII

XIV

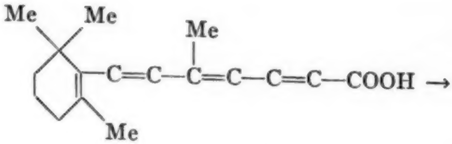
allylic rearrangements similar to that used by Oroshnik, point out an experiment where the hydroxy compound (XIII) was dehydrated to give the conjugated polyene XIV with an $\alpha$-ionone ring. From the nature of Oroshnik's reactions and the published value for the absorption maximum of his product they conclude that his compound may also have an $\alpha$-ionone ring.

A quite different type of synthesis is described by Arens & van Dorp (21, 22). They reacted $\beta$-ionone by the Reformatsky reaction with $\gamma$-bromocrotonate to give the hydroxy ester (XV) which was then dehydrated and saponified. The resulting unsaturated acid (XVI) was subjected to an interesting reaction

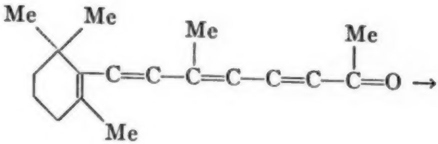
*Arens & van Dorp*



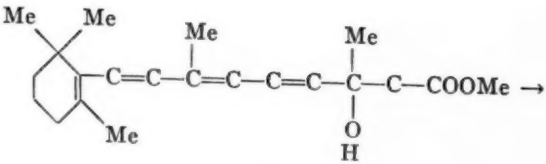
XV



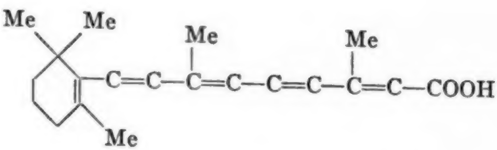
XVI



XVII



XVIII

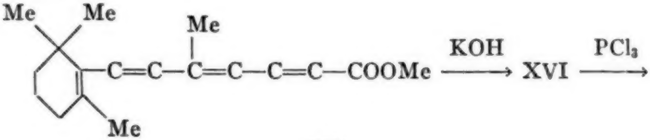


XIX

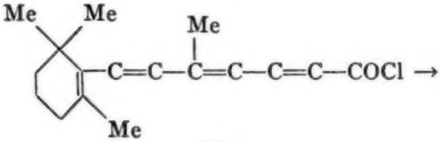
with methyl lithium to produce a methyl ketone (XVII). Another Reformatsky reaction with bromoacetic ester gave a hydroxy ester (XVIII) which on dehydration and saponification resulted in an important end product, namely the acid corresponding to vitamin A (XIX). When the sodium salt of this acid, dissolved in a buffer pH 10, was given orally to rats, 1 μg. of the material had a biological potency of 3.3 I.U. (23). Hence, the vitamin A acid seems to be about as potent as vitamin A itself. Arens & van Dorp (24) reported later that feeding A-depleted rats 1,000 μg. of vitamin A acid daily for seven days, or 3,000 μg. for three days, or injecting 10,000 μg. for three days, failed to produce a trace of stored vitamin A in the liver. The physiological implications of this are most interesting but should await discussion in later reviews.

Shortly after the publication of Arens & van Dorp, Karrer *et al.* (25) presented a paper which showed that they had been working on

*Karrer* et al.



XX



XXI

$\xrightarrow{\text{MeZnI}}$ XVII → XVIII → XIX

the same problem and had obtained almost the same results as Arens & van Dorp. In their reaction with β-ionone and bromocrotonic ester, they obtained the dehydrated ester (XX) directly upon working up their product. To make the necessary methyl ketone (XVII) they first made the acid chloride of the β-ionylidenecrotonic acid and reacted it with methyl zinc iodide. From then on their reactions were substantially the same as those of Arens &

van Dorp. Karrer *et al.* also made the corresponding derivatives from $\alpha$-ionone.

## Physiology and Nutrition

Table II is a correlation, somewhat rough because of the different methods of administration of the vitamin, of the effects of various levels of vitamin A on rats from the work of Lewis *et al.* (26), of Callison & Knowles (27), of Sherman *et al.* (28), and of Paul & Paul (29); on calves from the work of Lewis & Wilson (30); on sheep from the work of Peirce (31); and on humans from the work of Nylund (32).

TABLE II

Recent Studies of Conditions of Animals Fed Various Doses of Vitamin A

| Condition of Animals | Daily Dose (Units per Kg.) | References |
|---|---|---|
| *Night blindness, death* | | |
| Sheep | 17 (carotene) | (31) |
| *Night blindness* | | |
| Sheep | 50 (carotene) | (31) |
| *No night blindness, fair growth* | | |
| Rats | 20 | (26) |
| (*a*) fair longevity | 20 | (29) |
| (*b*) better growth and longevity than (*a*) | 40 | (29) |
| Calves | 32 | (30) |
| Sheep | 80 (carotene) | (31) |
| Humans | 28 | (32) |
| *Good Growth* | | |
| Rats | 100 | (26) |
| (*c*) Normal growth for 58 generations | 100 | (28) |
| Calves | 64 | (30) |
| *Good growth, plus* | | |
| Rats | | |
| optimal blood concentration | 200 | (26) |
| better growth and longevity than (*b*) | 200 | (29) |
| (*d*) longer life and reproductive period than (*c*) | 200 | (28) |
| *Appreciable liver reserves* | | |
| Rats | 300* | (27) |
| | 400 | (26) |
| longer life and reproductive period than (*d*) | 400 | (28) |
| Calves | 250 | (30) |

* A dose of 300 units/kg. for a 70 kg. man would be 21,000 units.

It is clear that the doses of vitamin A required for optimum health are many times larger than those needed to prevent night blindness or other easily recognized physiological symptoms. The importance of such conditions obtainable by higher dosage of vitamin A on rats such as length of life and other signs of well-being are great enough to justify more studies of the human requirements. Liver storage seems to be critical, and perhaps the micro-method of liver biopsy developed by With (33, 34) will be useful in studies of man and higher animals.

Sherman *et al.* suggested that, on the basis of their experiments, the human dose should be at least 10,000 units of vitamin A per day instead of the "official" requirement of 5,000 units per day. It would be easy to conclude from Table II that perhaps 20,000 units per day would be even better. Unfortunately, at the present, the relative biological ineffectiveness of carotene, and the short supply and high price of fish liver oils make it impossible for people of the world to use this much vitamin A. However, when vitamin A becomes more available, serious attempts should be made to increase the daily share that each person gets, particularly in backward countries.

Since many of the carotenes can be used to replace vitamin A in the nutrition of certain animals, the study of the relative equivalence of action of the carotenes as well as the nature of the conversion of carotene to vitamin A has been one of the most interesting fields of vitamin A physiology. Much precise data, which may turn out to be useful in studying this field, have been obtained by Deuel and co-workers who have biologically assayed some of the stereochemical isomers of the biologically active carotenes which have been isolated by Zechmeister & Polgár. Adequate supplies of tocopherol were added to the diet so that a deficiency of this material would not complicate the biological assay. The results of their determinations are summarized in Table III.

Some disagreement with their work has been noted by Kemmerer & Fraps (40). They found that neo-$\beta$-carotene U has only one-fourth the biological activity of $\beta$-carotene. In their work they made the interesting observation that there was change of neo-$\beta$-carotene U to neo-$\beta$-carotene B and $\beta$-carotene in the digestive tract of the animals and that all three carotenes could be also found in the feces.

With (41) has found that the conversion of carotene to vitamin A in the rat is a fast reaction which runs almost concurrently with the absorption process. Sexton *et al.* (42) found that carotene injected into the rat does not change into vitamin A at all. Some of the injected carotene is stored in the liver but a rat can die of vitamin A deficiency while these carotene stores were still fairly high. Their work shows that the conversion of carotene to vitamin

TABLE III

BIOLOGICAL ACTIVITIES OF CAROTENES FOR THE RAT

| Type of Carotene | Relative Activity | Reference |
|---|---|---|
| All trans-$\beta$-carotene | 100 | |
| Neo-$\beta$-carotene U | 38 | (35) |
| Neo-$\beta$-carotene B | 53 | (36) |
| All trans-$\alpha$-carotene | 53 | (35) |
| Neo-$\alpha$-carotene U | 13 | (35) |
| Neo-$\alpha$-carotene B | 16 | (36) |
| All trans-$\gamma$-carotene | 28 | (37) |
| Pro-$\gamma$-carotene | 44 | (37) |
| Cryptoxanthin | 57 | (38) |
| Neocryptoxanthin A | 42 | (39) |

A does not take place in the rat liver but probably takes place in the intestinal wall.

Stored or injected carotene appears to be utilizable by certain other animals. For example, Polozhintseva-Demina (43) found that carotene injections were quite efficient in improving the dark adaptation of vitamin A deficient children.

Carotene and vitamin A are readily transported in the organism by the blood serum even though these two compounds are not water-soluble. That a carrier complex is involved is suggested by Chalmers *et al.* (44) who found that in nonaqueous media carotene and vitamin A were destroyed by ionizing radiations with an ionic yield of almost unity; however, in human blood serum, vitamin A and carotene are protected in some way so that the ionic yield is reduced to 0.01. A study of the extractability of carotene and vitamin A from human blood plasma, which considered the effects of pH, solvents and salts, led Dzialoszynski

*et al.* (45) to conclude that the vitamin A and carotene in plasma are probably linked to an albumin.

While carotene may serve as a replacement of vitamin A for ordinary maintenance, in some cases it seems that it might not be suitable if it is required to build up liver stores of vitamin A. Bentley & Morgan (46) in studies of the nutrition of guinea pigs report that the liver stores were small when carotene was fed and could be made appreciable only by feeding vitamin A. With (41) found that to get appreciable stores of vitamin A in rats he had to feed five times the "optimum" dose, but it took twenty-five times the "optimum" dose of carotene to get storage. Wise *et al.* (47) found that newborn calves did not have much stored vitamin A whether their mothers had been fed on standard winter rations or on pasture grazing which provided an abundance of carotene in the prepartum diet. However, feeding 1,000,000 U.S.P. units of vitamin A daily to the dairy cows produced calves with significantly enhanced stores of vitamin A in the blood and livers.

Under certain conditions carotene might be more effective than vitamin A. For example, Pirie & Wood (48) found that rabbits which had been vitamin A deficient for six months or longer did not respond well to dosage with vitamin A in peanut oil. However, feeding carotene in the form of cabbage quickly restored the plasma vitamin A levels to normal.

In some tissues there seems to be antagonism between vitamin A and carotenoids. Rubin & Bird (49) found that feeding vitamin A will suppress the formation of carotenoid pigments in the leg of the fowl. This inhibition of pigmentation can also take place by a sufficiently large body store of vitamin A and thus is not exclusively an intestinal phenomenon. This antagonism is reminiscent of the well-known effect of feeding large doses of vitamin A to dairy cows; the carotene content of the milk will drop appreciably. This decrease in carotene can be partially protected against by feeding tocopherols, according to the work of Harris *et al.* (50).

It is not yet certain that some of the carotenes or carotenoids are not themselves directly used by certain animals and thus can be classified as vitamins. With (51) points out the comparatively high activity of β-carotene for hens and the unusually high activity of cryptoxanthin. From such considerations he suggests that cryptoxanthin itself is a vitamin A for the hen.

Calves that are severely deficient in vitamin A show an in-

crease in spinal fluid pressure accompanied by lower ascorbic acid contents of the blood and spinal fluid. Moore (52) showed that these two effects are not interrelated since the lowered spinal fluid pressure still occurred in vitamin A deficient calves even when ascorbic acid was injected or when its natural production is stimulated by chlorobutanol.

Frey & Jensen (53) found that the vitamin A reserves in the liver dropped rapidly when steers are put on fattening rations preparatory to marketing. Their data support the hypothesis made by Hickman (54) that in uncomplicated conditions the vitamin reserves of an animal on a vitamin-free diet will decrease at a constant fractional rate. Frey & Jensen's data recalculated by them in a private communication illustrate this as shown in Table IV.

TABLE IV

VITAMIN A RESERVES OF STEERS ON FATTENING RATIONS

| Vitamin A Reserve Units per Gm. Liver | Days in the Feed Lot | Decrease in Reserves per Forty-Day Period |
|---|---|---|
| 51.4 | 0 | — |
| 24.2 | 40 | 53 |
| 11.1 | 80 | 54 |
| 5.2 | 120 | 53 |
| 2.2 | 160 | 58 |

The clinical aspects of vitamin A are outside the general field of this review, but mention will be made of a few papers which had direct chemical and biochemical implications. Clausen *et al.* (55) found that patients suffering from celiac disease absorbed esterified vitamin A quite poorly after oral administration. Although the absorption of the esterified vitamin A is low, many children suffering from celiac disease can readily absorb vitamin A alcohol. The factors which prevent the proper assimilation of ester of vitamin A do not interfere with the subsequent esterification of the vitamin A for transfer in the blood plasma. Sobel *et al.* (56) found that children with the celiac syndrome could not absorb vitamin A from a blend of fish liver oils but received benefit from an "aqueous" solution of vitamins A, B, C, and D. Since many of

such preparations contain vitamin A alcohol, it may be that this work is quite parallel to that of Clausen & McCoord.

Aron *et al.* (57) reported from a study of ninety-two patients that the elevation of the body temperature to 105-106.5° F. by physically induced fever was followed by a depression of plasma vitamin A and carotene which was directly related to the duration of the fever. They suggest that this behavior of plasma vitamin A is the first biochemical indicator which can serve as a measurement of the intensity of action of physically induced fever on the human organism.

Ten years or more ago, it was often reported that patients with diabetes mellitus had carotene levels that were often above normal. Mosenthal & Loughlin (58) and Lambrechts *et al.* (59) found more recently that diabetics have normal carotene levels and conclude that there is no relation between the carotene level and the disease. Lambrechts suggests that the carotenemia of diabetes must have been of alimentary origin and that changes in food habits, especially in Europe during the war, were responsible for the change in the average values of this property.

Popper *et al.* (60) found that hypervitaminosis A judged by plasma levels was found in renal disease of the nephritic type. No evidence was obtained to indicate that liver damage contributed to the elevated vitamin A levels and they suggest that the destruction of the kidney parenchyma either causes retention of a protective substance or decreases the production of a substance destructive for vitamin A. Feeding an excess of vitamin A produces a much higher blood level in these nephritic cases than in normals.

## Vitamin $A_2$

Jensen *et al.* (61) found that while vitamin $A_2$ was somewhat toxic to rats in high doses, it had appreciable biological activity at the dosage levels ordinarily used in biological assay. A concentrate they prepared from Northern pike liver oil had a biological potency of 47,500 U.S.P. units per gm. and a value of 39.0 for E(1%, lcm.) ($35_2$ m$\mu$). Since this "conversion factor" had a value of over 1,000, it appears that vitamin $A_2$ has a large fraction of the biological activity of vitamin A judged by official growth response assay. Shantz *et al.* (62) carried out experiments to see if vitamin $A_2$ could be substituted for vitamin A in the tissues of the rat.

Vitamin A depleted rats were fed about 100 units of vitamin $A_2$ per day. The vitamin A storage in the liver was substantially all vitamin $A_2$ from the first week of the experiment; however, the vitamin found in the blood plasma after six weeks was mainly vitamin A. Apparently the vitamin $A_2$ had released some vitamin A from some of the rats' reserves. After twelve weeks of feeding, substantially all of the vitamin A in the blood was in the form of vitamin $A_2$ and the difference spectrum of visual purple of the eye had a maximum at 520 m$\mu$. Since the maximum for the visual purple (porphyropsin) of normal rats is at 505 m$\mu$ and that for the visual purple (rhodopsin) of fresh-water fish is at 535 m$\mu$, it is apparent that appreciable amounts of vitamin $A_2$ had entered in the rats' visual system. The rats in this experiment were somewhat below normal in growth and reproduction, which seems to confirm earlier work indicating the toxicity of high doses of vitamin $A_2$.

## Evaluation of Vitamin A Active Materials for Human Use

The information on the foregoing pages shows that the several substances that pass the biological assay for vitamin A may have widely different suitabilities as ingredients in foods and therapeutic preparations. The suitabilities of the different types have not been thoroughly investigated, but to stimulate study of this matter it is worthwhile to make the following suggestions regarding each:

Carotene—attractive color, low efficiency for humans, excellent for coloring margarine, butter and other foods; Vitamin A alcohol—often poor stability, good for "aqueous" preparations, especially for patients with celiac disease; Vitamin A ester—good stability and high biological efficiency, good for general use; Vitamin A acid—no storage in liver, might be useful therapeutically; Vitamin $A_2$—toxic in doses of effective size.

The above comparisons are not made with the intent of being either just or accurate, but they certainly contain enough truth to establish that a conventional rat bioassay does not in itself give enough information to evaluate vitamin A preparations for human use. Fortunately, if the chemical identity of the source of A-activity is not known from the history of the preparation, it can usually be determined readily by physical and chemical analysis.

## Biological Assay

The present official methods for the bioassay of vitamin A are not completely satisfactory with respect either to the standards or the procedures. Defects in the U.S.P. series of cod liver oils include their instability (63), their inability to be used as physicochemical standards, and the danger that each new reference oil will upset previously determined relationships. The series of International Standard carotene solutions should soon become reproducible from one preparation to another, but they are unstable, and, even more important, differ widely in chemical and physiological properties from the form of vitamin A used for human therapy and for dietary supplementation.

The recent production of pure vitamin A preparations, the free alcohol (64), the acetate (65), and other esters (65, 66), have made it seem desirable that a solution of one of these compounds be used as a standard. Various private recommendations, which have also been adopted in a recommendation of the Vitamin Committee of the American Oil Chemists' Society (67), are that the standard be a solution of pure vitamin A acetate at a potency of about 3,000 μg. per gm. of carrier oil and that this oil should be a refined and deodorized cottonseed oil, possibly fortified with a little more tocopherol. Such a product will show little if any interference with physicochemical tests and will have excellent stability compared with former standards. The committee further recommended that the preparation be put up in one-piece gelatin capsules because of the good keeping qualities of this type of product. The Vitamin Sub-Committee of the U. S. Pharmacopoeia is at the present time studying a preparation like that recommended by the Oil Chemists.

The diets used for the assay rats need to be better standardized, since variations in the vitamin E content and the nature of the protein are already known to influence the ratio of response between vitamin A and carotene. Using a vitamin A preparation as the basic standard will relieve most of these troubles. Gridgeman (68) has given an excellent summary of the information available on this subject as well as other aspects of the estimation of vitamin A.

## Physicochemical Assay

*Antimony trichloride reaction.*—Although some British workers (69, 70, 71) condemned the blue color procedure during the early 1930's, during the past fifteen years it has become a well accepted assay method for vitamin A, especially in food, feed, and physiological extracts. A fairly general method for using it with food products is given by Oser *et al.* (72), while typical recent methods for using it in feeds are given by Cooley *et al.* (73), and by Brew & Scott (74). The difficulties with the method used for the first ten years after Carr & Price discovered it in 1926 were due partly to inhibition effects which are now quite well understood and which are especially obnoxious in the case of products such as cod liver oil. The instruments used for measuring the color also contributed to the difficulties. The Lovibond Tintometer, which was given a semiofficial standing at first, has been shown to be unsuitable for the measurement of this particular shade of blue. Good results can be obtained with an instrument using a weak light source (75) and a direct color indication; a widely used photoelectric colorimeter is that designed by Evelyn (76).

A reaction similar in many ways to the antimony trichloride reaction, but requiring glycerol dichlorohydrin, has been pointed out by Feinstein (77) and by Sobel & Werbin (78, 79). The method is a little less sensitive, but the colored product is considerably more stable and the reagent much less corrosive than antimony trichloride.

One of the most significant developments in the use of colorimetric methods for vitamin A has been the adoption of the practice of calibrating the instrument with a known preparation of vitamin A instead of reporting the results in absolute terms such as blue values, L-values, cod liver oil units, etc. Unfortunately, the U.S.P. reference cod liver oils are of no help for this purpose, since even the unsaponifiable fraction seems to show noticeable inhibition effects. In the absence of any official vitamin A product for standardization, crystalline vitamin A, vitamin A acetate, and vitamin A naphthoate have been utilized. The pure vitamin products are somewhat unstable, and their very high potency makes them awkward to handle. For this reason, capsules con-

taining standardized samples of distilled fish liver oil have been very widely used, especially since Oser *et al.* (72) showed that such a concentrate is substantially free from materials which interfere with the ultraviloet absorption curve or the antimony trichloride reaction. The proposed new vitamin A standard, mentioned earlier, should be an excellent product for this purpose.

*Ultraviolet determination.*—It has been the general commercial practice for many years to determine the vitamin A potency of fish liver oils and concentrates by measurement of the ultraviolet absorption spectrum. The extinction coefficient at 325 to 328 mμ is taken to be proportional to the vitanim A potency. It is obvious that this method is unsatisfactory if the product being tested does not show the typical ultraviolet absorption curve of vitamin A. The first official notice of this effect appears in the contract form No. PDP76 for vitamin A oils and concentrates issued by the War Food Administration in 1944. They gave the following specifications for the spectrophotometric assay for vitamin A.

"The spectrophotometric assay on the whole oil dissolved in ethanol or isopropanol will consist of measurements of the extinction coefficient [E (1%, 1cm.)] at the following wavelengths: 300 mμ, 328 mμ, and 350 mμ. The ratio of E(300 mμ)/E(328 mμ) shall not be more than 0.73 and the ratio of E(350 mμ)/E(328 mμ) shall not be more than 0.65. The potency will be calculated by multiplying the value of E(1%, 1cm.) (328 mμ) by the conversion factor 2000."

The main reason that the ultraviolet method for assay has been preferred over the blue color method is that it is more precise. Even this precision has been none too good in the past. Coward *et al.* (80) calculated that the coefficient of variation of the ultraviolet absorption measurements may have been as large as 20 per cent. Wilkie (81) in a collaborative test found coefficients of variation of about 6 per cent. Rawlings & Wait (82) using a Beckman spectrophotometer and taking into account all the errors of procedure were able to show that a coefficient of variation of about 0.2 per cent could be obtained on routine laboratory operation. Intralaboratory agreement on assays for commercial purposes in this country during the last year or so have confirmed that E-value determinations with modern instruments are reasonably precise. The basic question remains of what conversion factor to use for calculating the vitamin A potency from the extinction

coefficient. Hume (83, 84) and Irwin (85) have described an English study of collaborative bioassays analyzed by extremely careful statistics. They found that an extinction coefficient of unity corresponds to 1740 I.U. of vitamin A. Müller & Reinert (86), from their work in Switzerland, report a "conversion factor" of 1700. In the United States the conversion factor is usually found to have a value of about 2000 whether or not the vitamin A product is being tested against fresh U.S.P. reference oils or the International Standard carotene. This may be due to the instability of both standards, a situation which will be clarified by the new policy of having the International Standard made up at frequent intervals in each country.

Although the precision of E-value determinations is quite good with certain instruments, there still remains the difficulty of making certain that the readings are accurate. There is no simple way for a routine control laboratory to check up on the actual amounts of incident and transmitted light on the solutions being tested. For this reason, recourse has to be made to stable products whose ultraviolet absorption characteristics have been carefully established by physical laboratories. Vandenbelt *et al.* (87) present data concerning the absorption spectra of such classical substances as potassium nitrate and potassium chromate. Unfortunately, neither of these standards shows the same absorption curve as does vitamin A. Kreider (88) suggests the use of 2-phenolazo-*p*-cresol as a photometric standard since it shows an absorption band much like that of vitamin A and is very stable. In the past there has been a tendency to use the U.S.P. reference oils as spectrophotometric standards. The peculiarities in the absorption spectra of these oils and their instability have made this practice unsatisfactory, but it seems likely that a suitable biological reference standard could be quite useful as a spectrophotometric standard.

Most chemists specializing in vitamin A believe now that a rigorous physicochemical assay procedure should be set up on an official basis. Wilkie (89) recommends that the value of E(1%, lcm.) (328 m$\mu$) of the unsaponifiable fraction, provided the shape of the curve is suitable, be multiplied by a factor of 2000 to give an official potency. This is substantially the same recommendation made by Gridgeman (68). Metcalf (90) recommends that vitamin A potencies determined by a physicochemical method be expressed in metric terms such as mg. of vitamin A per gm. of oil.

The Vitamin Committee of the American Oil Chemists' Society (67) recommends that a physicochemical assay procedure be set up on an official basis and consist of the following factors: purification sufficient to give a typical vitamin A absorption spectrum, calculation of the potency from the value of E(1%,1cm.) (328 mμ), and confirmation by the antimony trichloride reaction.

## VITAMIN E

### Chemistry

A significant development in the chemistry of vitamin E has been the discovery by Stern *et al.* (91) of a fourth natural tocopherol which they have called δ-tocopherol. The chemical and physical properties of δ-tocopherol indicate that it is 8-methyl tocol. It is thus the first mono-methylated tocopherol isolated from natural sources. Of the total mixed tocopherols in soybean oil about 30 per cent occurs as δ-tocopherol. δ-Tocopherol is somewhat more active as an antioxidant than γ-tocopherol but seems to have a low biological activity in preventing sterility in rats.

### Physiology and Nutrition

It is becoming increasingly apparent that many of the biological properties of the tocopherols depend upon their ability to act as antioxidants. While many reports of the antioxidant action of tocopherol on fats *in vitro* have been made in the last fifteen years, the antioxidant action of tocopherols in the animal was brought into prominence by the work of Hickman and associates (92 to 97) when they showed that the biological response of vitamin A and carotene was very greatly influenced by the presence of tocopherols (and to some extent by other antioxidants) even on diets official for vitamin A assay. Part of Hickman's work has been rather thoroughly checked by Lemley *et al.* (98) who confirmed his findings that rats fed a U.S.P. vitamin A deficient diet (containing olive oil or cottonseed oil as the "vegetable" oil) plus 2.04 units of vitamin A per day showed greater increases in weight when tocopherols were fed. Increasingly larger doses of vitamin A showed lesser benefits from the tocopherol administration, indicating that the main effect of the antioxidant was to aid in the utilization of this vitamin. In addition, these investigators found that the injection of a water-soluble preparation of tocopherols increased the growth response to vitamin A. This, combined with Moore's

(99) findings that the administration of tocopherols increased the length of storage of vitamin A in the liver shows that the antioxidant effect on vitamin A is present in the tissues of the rat as well as in the gastrointestinal tract.

Other constituents of the diet which exert a synergistic action on the antioxidant activity of tocopherol *in vitro* can often exert a similar effect *in vivo*. Tomarelli & György (100) found that rice bran extract will exert protective effects on carotene both *in vitro* and in a biological test similar to a vitamin A bioassay. Scharf & Slanetz (101, 102) found that phospholipids have a favorable effect on the utilization of vitamin A even in the presence of adequate tocopherol. This is reminiscent of the *in vitro* synergism given by the phospholipids.

The importance of the stability of a vitamin A supplement was emphasized in a paper by Chevallier & Manuel (103) which probably was written without knowledge of the work on tocopherol carried out in this country. By measuring the oxidative stability in a Warburg apparatus, they found that the stabilities of the fish liver oils they worked with varied from fourteen hours to eight days. When these oils were fed to guinea pigs the portions of the ingested vitamin A stored in the liver varied directly with the stability measured by the Warburg apparatus.

Interesting evidence that tocopherol is not the only fat soluble antioxidant in animal tissues has been discovered by Dubouloz *et al.* (104, 105). They found three "antioxygens" which have many properties similar to the tocopherols but may be differentiated most easily by the fact that their ultraviolet absorption maxima are at about 270 m$\mu$.

A protective effect given by vitamin E to rats made diabetic by alloxan injection has been found by Clausen *et al.* (106). Such alloxan diabetes quickly depletes the stores of vitamin A, even when the rats are fed a normal amount of vitamin E; 0.5 mg. extra vitamin E daily lessens the loss of vitamin A and prolongs the life of the experimental animals.

Hemorrhagic kidneys in choline-deficient rats were not prevented by inositol, despite its lipotropic action in the liver. But, Handler (107) found that a combination of inositol and tocopherol gave some protection. The biological effect of this combination will be mentioned again.

Hove & Harris (108) have found that tocopherols greatly

increase the effectiveness of less than optimal doses of methyl linoleate in curing essential fatty acid deficiencies in rats. The response to tocopherol of rats fed over 80 mg. of linoleate per day is neglible which shows that the primary function of the tocopherols is to increase the quantity of linoleate available to the rat. These authors also found that pyridoxine is a fairly powerful antioxidant and suggest that the sparing action of pyridoxine on essential fatty acid found by Salmon (109) may be due to its antioxidant action.

A related effect on the action of tocopherol on dietary linoleic acid has been found by Fraenkel & Blewett (110) who found that wheat germ oil was essential for the proper growth and emergence of the flour moth, *Ephestia kuehniella*. The wheat germ oil could be replaced by linoleic acid and $\alpha$-tocopherol, and in this combination the $\alpha$-tocopherol could fairly satisfactorily be replaced with other antioxidants such as gallic acid esters or ascorbic acid.

Increasing the fat (lard) content of the diet of rats decreases their survival time under conditions of anoxic anoxia. Tocopherol is effective in increasing the ability of the animal to survive under conditions of anoxia as described by Hove *et al.* (111) which indicates some effect of tocopherol in regulating oxygen metabolism.

The stabilization of body fat of pigs by dietary tocopherol has been reported by Watts *et al.* (112) and Chipault *et al.* (113). The latter authors concluded from their experiments that the keeping quality of carefully processed hog fats depends primarily on their contents of unsaturated fatty acids and of tocopherol and further that the tocopherols are the only natural direct inhibitors of oxidation present in these fats.

When dairy cows are fed a daily supplement of 500 to 1,000 mg. of mixed natural tocopherols, the milk fat output is increased by about 20 per cent. In these experiments, Harris *et al.* (114) also noticed that the decrease in carotene concentration in the milk due to large intakes of vitamin A is prevented in part by a tocopherol supplement. The mechanism of these functions of vitamin E is not clear as yet, but certainly deserves much further study on both physiological and economic grounds.

Relations between vitamin E and the fat metabolism of chicks have been shown by study of the occurrence of exudative diathesis and encephalomalacia. The appearance of these conditions, which occur when chicks are fed certain diets low in vitamin E, depends

on the nature of the accompanying fats. Certain generalizations can be made, but due to the numerous experiments with different diets, the original literature [a good recent guide is Dam (115)] must be carefully studied for details. With no fat there are no symptoms within thirty five days, but with 5 per cent cod liver oil or cod liver oil fatty acids or linseed oil fatty acids, the exudative diathesis appears in two weeks. Hog liver fat induced encephalomalacia. When the fatty acids of hog liver fat were fractionated into a saturated, an unsaturated, and a middle fraction, and then fed, the saturated fraction (as well as oleic acid) had little effect. The unsaturated fraction produced encephalomalacia and death in all animals within two weeks and the middle fraction gave both exudative diathesis and encephalomalacia. Peroxides usually appear in the body fat at about the same time the symptoms appear. Dam shares the increasingly popular view that the simplest and most direct explanation of the symptoms is that they are due to tissue damage caused by abnormal oxidation products of the unsaturated fatty acids. The symptoms and, hence, the abnormal oxidation, are always prevented by vitamin E. There remain to be explained the occasional spontaneous cures without vitamin E and the slight but appreciable relief given by lipocaic and by inositol. Of course, these materials are both important in fat metabolism and the latter has been reported by Milhorat & Bartels (116) to be active with tocopherol in the treatment of progressive muscular dystrophy. Milhorat's (117) finding that injected tocopherol phosphate helped this disease indicates that inositol aids the assimilation of tocopherol.

Dam & Granados (118) have found that rats placed on vitamin E deficient diets which included 20 per cent cod liver oil did not show exudative diathesis but instead showed a striking depigmentation of the enamel of the incisors. They established that this effect was due to the unsaturated fatty acid fraction of the cod liver oil. Vitamin E prevented the depigmentation.

Following Dam & Mason's discovery (119, 120) that vitamin E deficient rats on diets high in cod liver oil showed a discoloration of the adipose tissue resulting from the deposition of an acid-fast pigment, Filer *et al.* (121) showed that lard produced the same effect. Similar diets, but with linseed oil methyl esters caused much pigment to deposit in the fat depots, and arachidonic acid in the diet gave poor growth, anemia, and early death with atrophic

adipose tissue. Other differences in the actions of these fats are reminiscent of the actions of the different fats for chicks which indicated that the uncontrolled oxidations of the different acids will produce symptoms in different organs.

The pigment causing the discoloration of the adipose tissue seems to be the same as that discoloring the smooth and skeletal muscle of vitamin E deficient rats and as that designated by Victor & Pappenheimer (122) as ceroid pigment in the liver of rats maintained on a low-protein, vitamin E free diet. Extensive histological studies by Mason & Emmel (123) give further evidence that it is an abnormal metabolite (or a normal metabolite having but a transitory existence in the vitamin E sufficient rat).

Mason & Filer (124) have presented a review covering the evidence showing that the level and type of dietary fat (i.e., unsaturation, "sweetness," and antioxidant content) markedly influence the tocopherol requirement of experimental animals whether the requirement is measured by the prevention of sterility, muscular dystrophy, or deposition of acid-fast pigment in body tissue.

Most of the preceding discussion of the place of vitamin E in physiology has shown a linkage of this vitamin with fat metabolism. When Dam (125) found that vitamin E prolonged the lives of rats placed on a diet fatally low in protein, he offered no explanation for this effect. Recent work by Hove *et al.* (126) shows that stomach lesions are produced in rats by deficient diets whether low in protein, in essential fat acids, in pyridoxine or simply in calories. In all cases these lesions are prevented by daily doses of $\alpha$-tocopherol and the authors suggest that there might be a relation between vitamin E and amino acid metabolism. Another relationship between vitamin E and gastric ulcers in rats was shown by Jensen (127) who found that the stomach ulcers produced by diets low in vitamin A could be prevented or cured with vitamin E.

Some of the action of vitamin E must be the influencing of, or taking direct part in, enzyme systems. This field is being actively studied but will be mentioned only briefly because of the present uncertainties of the work. Basinski & Hummel (128) have presented evidence to disprove Houchin's earlier report (129) that there was an increased rate of enzymatic oxidation of succinic acid by skeletal muscle in nutritional muscular dystrophy, although their

experiments do not preclude the possibility that water-soluble tocopherol esters may play an important role as a natural governor system in keeping the succinic dehydrogenase under control. Torda & Wolff (130) find that vitamin E has a direct action on the production of acetyl choline in minced frog brain; for example, as little as 0.3 $\mu$g. of $\alpha$-tocopherol in 100 mg. of brain increased the production by 60 per cent. Morgulis & Jacobi (131) present evidence that the increased rate of oxygen uptake in dystrophic muscle is due to enhanced adenosine triphosphatase activity. Govier *et al.* (132) found that digitoxin greatly increased the oxidation of lactate by E-deficient guinea pig heart muscle homogenates. This increase can be prevented by $\alpha$-tocopheryl phosphate, genates. This increase can be prevented by $\alpha$-tocopheryl phosphate, and such an increase is not exhibited by normal heart tissue. A study of the effect of vitamin E on reproduction by Gaehtgens (133) led him to conclude that the remarkable interference of E-deficiency with reproduction is of a nonspecific nature; that is, there is no selective relation with the sex hormonal systems. The disturbances must be due to more generalized effects such as dislocations in carbohydrate metabolism and in phosphorylation processes.

The place of vitamin E in human and farm nutrition is a problem still requiring the best attention of scientific workers. Many instances have been reported in the past of domestic animals suffering ill effects from "normal" diets which turned out to be deficient in this vitamin. In the last year reports have become available which describe instances where, due to vitamin E deficiency, mares have had breeding troubles (134) and sheep have had the stiff lamb disease (135, 136). Supplements of tocopherol have had favorable effects on decreasing the mortality of laying hens (137) and on the general health of domestically raised foxes and minks (138).

A survey by Harris *et al.* (139) of the blood levels of tocopherol in an area of endemic malnutrition in southern United States showed that the tocopherol levels of the nutritional clinic patients averaged 25 per cent less than those of a "normal" group living in the same area.

Hickman & Harris (140) estimated, using known requirements of laboratory animals, that the normal adult of average size will require about 30 mg. of mixed natural tocopherols per day. Quaife

& Harris (141), in their study of American foods, found that the country's foodstuffs did, indeed, furnish an average of 15 mg. of *d*, $\alpha$-tocopherol and an equal quantity of a mixture of the $\beta$-, $\gamma$-, and $\delta$- forms per person per day; but the varied distribution of tocopherol in food makes it possible that those whose diets are low in vegetable oil products may get an amount of tocopherol that is a very small fraction of the average.

The strong but as yet indirect evidence that vitamin E is important in practical nutrition may be summarized in this simplified manner: (*a*) Cases have been found where traditional diets for domestic animals are low enough in vitamin E to injure seriously their health, and vitamin E must be added to the purified or synthetic diets used for studying the nutrition of laboratory animals; (*b*) The "requirement" of vitamin E by humans, estimated from the "requirement" of vitamin E by laboratory animals, is about equal to the average quantity available in all food, but the unequal distribution of this vitamin makes it seem likely that many persons get very much less than the "requirement"; and (*c*) A group of underprivileged persons was found to have an average tocopherol blood level appreciably below that of normals.

The problem of studying the human requirements of vitamin E is complicated by the possibility that the need for this vitamin may be increased at certain periods during development. For example, Kaunitz (142) has found, by measuring growth and testicular development, that rats have a critical requirement during the third week of life.

Like the rat fetus, the human fetus may have a very low content of tocopherol compared with that of the mother. It has been found by Varangot *et al.* (143) and by Kofler (144) that the blood level of tocopherol of the human fetus is much lower than that of the mother. The recent study of this subject by Straumfjord & Quaife (145) suggests that there may be a natural mechanism by which the entry of tocopherol in the fetus is encouraged. They found as did the other workers that the vitamin E level of cord blood of infants at birth is one-fifth that of the level in the venous blood of the mothers. But the average plasma vitamin E level of mothers at term is 65 per cent higher than that of nonpregnant women.

The clinical importance of vitamin E with respect to reproductive troubles and muscular degenerations is still of interest and

is still disputed. Since a full discussion of the clinical field is outside the limits of this article, attention will be drawn only to two papers. Skelton & Shute (146) found that experimentally produced purpura in dogs as well as human purpura could be improved or cured with 200 to 400 mg. of $\alpha$-tocopherol daily. Vogelsang & Shute (147), while studying this treatment of purpura, noticed an improvement in the heart condition of certain subjects. Further studies of the treatment of heart patients showed that tocopherol was distinctly beneficial to patients having congestive heart disease and the anginal syndrome; exercise tolerance was increased and anginal pain was diminished or abolished. This stimulating publication has caused other workers to make similar studies, but while these are being carried out, it is interesting to speculate on the high tocopherol content of normal heart tissue, the earlier mentioned difference in enzyme action of normal and E-deficient heart tissue, the change in blood pressure in E-deficient rats found by Telford *et al.* (148), and the report of Gullickson & Calverley (149) that many cattle on vitamin E-free rations died suddenly from heart failure.

## Assay

No recent developments have changed the accepted technique for the biological assay by the rat antisterility method. This has turned out to be a reasonably good biological method both with respect to precision, accuracy and convenience. However, there are many other biological functions of vitamin E, and it may well turn out that the measurements of some of these activities will be adopted as assay methods, at least for certain specialized purposes. Table V summarizes the recently reported quantitative biological effects of the different tocopherols.

The physicochemical assay of vitamin E has been a difficult problem due to the lack of specific reactions and of easily usable spectral properties of the tocopherols. Recently two suggestions for assays have been made based on the oxidation of the tocopherols to the *o*-quinone, and the reaction of this quinone with other agents to give a characteristic colored product. Kofler (144) reacted the tocopherol quinone with *o*-phenylenediamine. The product can be easily measured by fluorometric procedure. Chipault *et al.* (113) reacted the quinone with leucomethylene blue to give methylene blue. While these two methods have very good sensitivity, the accuracy and convenience are seriously hindered by the neces-

sary purification steps needed, and resort to such methods will probably be limited.

Most of the recent work in this field has been to modify the original Emmerie-Engel reaction which depended on the measurement of the red color produced when tocopherol reacts with a mixture of ferric chloride and $\alpha,\alpha$-dipyridyl.

Kaunitz & Beaver (155) have shown that many fat constituents inhibit this reaction and they give directions for correcting this effect. Tošić & Moore (156) recommend that the oils be saponified

TABLE V

COMPARATIVE POTENCIES OF THE TOCOPHEROLS*

| | Relative (Approximate) Biological Potencies | | | | | | |
|---|---|---|---|---|---|---|---|
| | $d,\alpha$ | $dl,\alpha$ | $d,\beta$ | $dl,\beta$ | $d,\gamma$ | $dl,\gamma$ | $d,\delta$ |
| Rat | | | | | | | |
| Antisterility (150) | 100 | 67 | 33 | 16 | 1 (153) | <1 | <1 (91) |
| Weight gain (151) | | 100 | | 25 | | 19 | |
| Vitamin A protection (93) | 100 | | 100 | | 100 | | 100 (154) |
| Rabbit | | | | | | | |
| Creatinuria (111) | 100 | | | | 15 | | |
| Chick | | | | | | | |
| Exudative diathesis (152) | | 100 | | 6 | | | |

* Figures in parentheses are literature references.

and subjected to a chromatographic purification before the Emmerie-Engel reaction is carried out. Their method admittedly is good only for $\alpha$-tocopherol. However, by coincidence, the fraction of $\beta$-tocopherol measured by their method is not far from its relative biological activity.

A specialized technique for determining the tocopherols in blood plasma has been worked out by Quaife & Harris (157) who use hydrogenation to inactivate the carotenoids and other interfering substances. A simplified apparatus for this purpose has been described by Quaife & Biehler (158).

Baxter & Stern (159) point out that acetic acid is inadvisable as a solvent for the Emmerie-Engel reaction if tocopherols other

than $\alpha$-tocopherol are present. They also show that the newly recognized $\delta$-tocopherol is not correctly assayed by any of the previous modifications of the Emmerie-Engel reaction. A very careful control of the time of the reaction as well as the concentration of the reagents is necessary to get a reliable estimate of tocopherol content.

A highly important problem is the determination of each tocopherol in mixtures of tocopherols. Hove & Hove (160) have developed a method which will estimate $\alpha$-tocopherol in mixtures of $\alpha$- and $\gamma$-tocopherols by carrying out the Emmerie-Engel reaction in acetic acid at two different temperatures. Fisher (161) has developed a method for determining $\gamma$-tocopherol in mixtures of $\alpha$- and $\gamma$-tocopherols. It depends upon the reaction of tocopherols with nitric acid under such conditions that the $\gamma$-tocopherol gives rise to a red quinone while the $\alpha$- form produces a yellow product.

Weisler *et al.* (162) have investigated the problem which is met with when $\delta$-tocopherol is recognized as an important constituent of the tocopherol fraction in many vegetable oils. Starting with the method of Quaife (163), they studied the possibility of reacting $\gamma$- and $\delta$-tocopherols with other diazonuim salts. They found that diazotized *o*-dianisidine is an especially suitable reagent for coupling with $\gamma$-tocopherol. A further study showed that while this reagent also reacted with $\delta$-tocopherol, the extent of reaction with the two tocopherols differed so much at different values of pH that the authors were able to devise a reasonably simple method which would give the content of both $\gamma$- and $\delta$-tocopherols in mixtures of tocopherols. At the present time a preliminary concentration procedure, e.g., saponification, is necessary in working with preparations of low tocopherol potency such as vegetable oils.

## VITAMIN D

### Chemistry

Definite progress has been made on methods for the physicochemical assay of vitamin D. Ewing *et al.* (164) developed a physicochemical method for the determination of vitamin D in fish liver oils. They used a combination of chromatography and the antimony trichloride reaction. This work has been followed up by a description of procedures for determining the vitamin D content of oil solutions of irradiated ergosterols by Ewing *et al.* (165). DeWitt & Sullivan (166) have also described a procedure

for the spectrophotometric estimation of vitamin D in pharmaceutical products based on the antimony trichloride reaction and a chromatographic procedure depending on fluorescence in the presence of ultraviolet light to distinguish the chromatographic adsorption bands. Another method for the determination of vitamin D was discovered by Schaltegger (167). He used the formation of a colored product by a sterol and an aromatic aldehyde in the presence of a strong acid. By choosing different aldehydes and by other chemical manipulations he was able to determine vitamin D in the presence of numerous interfering sterols and other compounds.

## Physiology

Several recent papers (168 to 171) have shown that the efficiency of vitamin $D_3$ was often higher than the vitamin D in cod liver oil for promoting calcification in turkey poults. Matterson *et al.* (172) have studied the relation of the source of the phosphorus to this effect. Their experiments showed that when the phosphorus in the diet for the turkey poults was mainly inorganic phosphorus, the vitamin D from activated 7-dehydrocholesterol and from cod liver oil would produce approximately equal calcification in poults. However, if much of the phosphorus was present as phytin, that is, from cereal sources, then the vitamin $D_3$ was considerably higher in efficiency than the cod liver oil vitamin D. This work explains most of the discrepancies on this subject in the past literature.

In a study with turkey poults of the efficiencies of the vitamin D from cod liver oil, salmon oil, and irradiated 7-dehydrocholesterol, McGinnis & Evans (173) found that the salmon oil and irradiated 7-dehydrocholesterol were considerably more effective chick unit per unit than reference cod liver oil. Thus the vitamin D in salmon oil must be different chemically from that in reference oil. The complexities of the physiological action of the natural sources of vitamin D make it advisable that the next reference standard for vitamin D be made from a synthetically produced vitamin D of known chemical nature.

Among miscellaneous discoveries in the vitamin D field, it is worthwhile to mention that Gridgeman (174) has quite well established that on rats vitamin $D_3$ is 1.31 times as active as vitamin $D_2$. Glazener *et al.* (175) have found that there is an abnormal blackening at the base of the feathers of New Hampshire

chicks developed as a result of vitamin D deficiency and this could be prevented by feeding activated animal sterols. It would be indeed valuable if a selected strain of animals could be used to give visible indications of vitamin D action during a biological assay.

Irving (176) found that vitamin D deficient rats on a diet with calcium to phosphorus ratio of 5.6 would show a healing of bones when changed to a diet with a ratio of 1.9. This healing was somewhat different from that produced by feeding vitamin D while holding the animals on the high ratio diet. When D deficient rats on a low calcium phosphorus ratio of 0.26 were changed to a diet with a ratio of 1.9, the healing of their teeth closely resembled that of rats held on a low calcium phosphorus ratio but fed vitamin D.

Irving (177) and Weinmann & Schour (178) reported that rats on a Steenbock and Black type diet showing marked rickets would, if their food was sharply restricted, show healing, judged by their incisor teeth, in the same manner as if vitamin D had been administered in the diet.

## A NEW FAT-SOLUBLE VITAMIN?

Boer *et al.* (179) have found that on a diet containing all the known fat-soluble vitamins rats grew appreciably better when fed summer butter than they did when fed winter butter or rapeseed oil. Fractionation of the summer butter by vacuum fractional distillation showed that the active ingredient was a fraction consisting apparently entirely of vaccenic acid, an isomer of oleic acid with double bond between the $C_{11}$ and $C_{12}$ carbon atoms. When this fraction was added to winter butter or to rapeseed oil, the growth of rats fed this diet was practically equal to that of the rats fed summer butter.

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# WATER-SOLUBLE VITAMINS[1]

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In reviewing accomplishments in the field of water-soluble vitamins for the year 1946, various individuals will differ in their decisions on what to include. Those interested in clinical applications will place more emphasis on the treatment of anemia with folic acid, or the reliability of corneal vascularity as an index of nutritional state, while those with other specialties will be tempted to emphasize such metabolic aspects as the isolation of the precursors of choline. Certain studies which involve vitamins belong more to the sections on nutrition, bacterial metabolism, or intermediary metabolism and can only be alluded to here. The reader will find that many of the minutiae of new investigations can be traced much more rapidly and to better advantage in *Chemical Abstracts* than in this review. In the limited space at our disposal more emphasis will be placed on discussion of what broad fundamental advances have been made in 1946, rather than on efforts to treat the subject exhaustively.

The past year has seen some basic advances in our knowledge about water-soluble vitamins. One of the most outstanding was the elucidation of the structure of folic acid, and the use of this substance successfully to treat pernicious anemia and related macrocytic anemias. Another was the demonstration of a nutritive essential in certain proteins which was not an amino acid, and hence the finding that the time-honored "vitamin-free" casein really supplied more than essential amino acids in experimental diets. A third has been the re-evaluation of the etiology of pellagra, from which has emerged an insight into the interrelationships of nicotinic acid and tryptophane as well as an appreciation of the multiplicity of factors which may be involved in a naturally occurring deficiency disease. Still other basic advances will appear as we turn now to a consideration of the individual vitamins.

## Ascorbic Acid

Studies with this vitamin during 1946 have dealt mainly with refinements of methods of determination (1, 2, 3, 5, 8, 197) and in

[1] This review covers the period from December, 1945 to December, 1946.

applications of these procedures to estimation of the vitamin in natural products. Thus, a survey of the effects of soil, climate and season on the ascorbic acid-content of various strains of cabbage was made (4), paralleling an older study of tomatoes. Analysis of plants of central Asia (6) indicated that the *Rosaceae* and *Papilionaceae* were the richest sources. A sex difference in the vitamin C levels of rats has been found in that the blood of females was observed to contain only about half as much ascorbic acid as that of males (7).

The problem of the precursors for the synthesis of ascorbic acid in rats has been pursued further by the use of narcotics such as chloretone to stimulate formation of the vitamin. By maintaining animals on a very low protein diet, and noting the excretion of ascorbic acid resulting from administration of various amino acids, and chloretone, Roberts and Spiegel concluded that of all compounds tested, methionine and cystine gave rise to the greatest amounts of the vitamin (9). Similarly, Roy *et al.* (198) found that adequate thiamine nutrition was a prerequisite to chloretone stimulation of ascorbic acid synthesis. The observations of Kennaway & Daff (199) correlate nicely with this because they found that thiamine or riboflavin deficiency of mice led to reduced liver stores of ascorbic acid.

## Thiamine

In connection with this vitamin a group of observations has been made recently which may indicate a breach in the solid belief that the vitamins, regardless of what may be said about sex hormones or other biologically important compounds, really are very specific, and that not even closely related chemical substances possess activity. Thus Polonovski *et al.* (10) and Busnel *et al.* (11) reported that a pterin-like compound, fluorescyanine, extracted from fish scales was as active in thiamine depleted animals as was the vitamin itself. It was even claimed that the same compound could replace riboflavin in the diet of the rat (10). Synthetic isoxanthopterin carboxylic acid and several other pterins possessed somewhat less, but still considerable potency (10, 13). Despite the activity of fluorescyanine in replacing thiamine for the nutrition of rats, it was unable to serve as the vitamin for the growth of a flagellate, *Polytomella caeca* (12). This rather puzzling case might be dismissed as a vagary of nature were it not that analogous examples have been reported. One of these is that pantothenic acid will

replace thiamine and cholic acid in the promotion of growth of the bacterium *Clostridium botulinum* (14). It may be that the heterologous substances such as fluorescyanine exert their action by sparing the vitamin much as fat spares thiamine, but the need for more study is apparent.

The form in which thiamine occurs in yeast has been the subject for study and debate. One school has claimed that the majority of the vitamin occurs as the disulfide formed by cleavage and oxidation of the sulfur of the thiazole ring (15). The pyrophosphate of this substance may also occur. During fermentation of glucose it is said that the disulfide is reduced to thiamine or cocarboxylase (16). This view has been disputed (17) with the statement that at most, only small amounts of the disulfide occur in yeast.

Experiments have been continued with naturally occurring antithiamines. The thiaminase of aquatic animals, an enzyme which splits thiamine at the methylene bridge (18) has been investigated further with respect to mechanism of its action (19). Krampitz & Woolley (18) had shown in 1944 that in the case of carp two processes were involved, the first of which was the cleavage of the thiazole moiety from the vitamin and the formation of a derivative of the pyrimidine part. A second reaction then liberated the free pyrimidine from this complex. Hennessy *et al.* (19) have now shown that only the first process takes place in clams, and they have isolated the pyrimidine derivative which is formed. During active fermentation of glucose by yeast this substance may be caused to react with the free thiazole portion of thiamine to yield back the vitamin. Purification of the carp enzyme has been described (20).

A second substance antagonistic to thiamine has been indicated to occur in certain poisonous ferns. Indeed, the toxicity of the plants for rats was strikingly counteracted by administration of thiamine, and for this reason an antagonistic principle was believed to exist in the ferns (21).

Studies have been continued in efforts to show that thiamine, aside from its relationship to the classical manifestations of deficiency, exerts an influence on health and on resistance to diseases. Cooperman *et al.* (22) have continued the study, first made by Foster *et al.* (23), of the relationship of thiamine restriction to susceptibility to experimental poliomyelitis. They extended the study to a new species, and reported that with day-old chicks a

partial thiamine deficiency made the birds slightly more susceptible to paralysis by the virus, while, curiously enough, with two weeks old animals, the deficiency conferred a slight resistance. Guggenheim & Buechler observed that thiamine deficiency in mice resulted in increased ease of growth of *Salmonella* in certain of the organs during the two days following inoculation (24). They interpreted these observations to mean that the deficiency made the mice more susceptible to the infection. However, it was not demonstrated that the incidence of disease, rather than multiplication of the organisms, was changed by the vitamin deprivation. Harrell concluded that administration of thiamine to children in an orphanage (25) brought about favorable changes in response to certain mental and physical tests which were applied several months after treatment was begun. In all these reports the magnitude of the differences attributable to the action of the vitamin made it necessary to use large numbers of individuals in order to establish the significance of the results.

Although thiamine or its component thiazole and pyrimidine parts is a growth factor for many microbial species, Lankford & Skaggs (26) have found that for the gonococcus, thiamine is practically inactive whereas cocarboxylase is a nutritive essential.

## Riboflavin

Several noteworthy technicological developments for this vitamin have been made. One of these has been the discovery that fungi, such as *Eromothecium ashbyii*, synthesize large amounts of riboflavin (27) and that from culture filtrates of the organism the vitamin may be isolated in pure form in a single step (28). To do this the culture filtrate is subjected to the reducing action of growing bacteria. The riboflavin is thus precipitated as an insoluble red reduction product, which may be filtered off and converted to the vitamin by exposure to air. Surely, to those who have used the large number of steps previously required to isolate this vitamin from natural sources, the simplicity of this new process will be of interest. Another development has been the production of readily soluble and biologically active esters of riboflavin with succinic acid (29). One of the two carboxyl groups of the acid was left free to form a soluble sodium salt.

Although the microbiological method for the quantitative estimation of riboflavin has continued to enjoy popularity, the

accuracy of the fluorometric procedure has been improved by Scott *et al.* (30), so that this method now recommends itself because of the shorter time required as compared with the microbiological procedure. Oxidation with hydrogen peroxide has been used to remove interfering pigments before fluorometry of riboflavin, and so the observations of Leviton (31) are of interest. In the presence of appropriate concentrations of ferrous ion he found that the vitamin was readily destroyed by the peroxide, while in the absence of ferrous ion there was no loss. This was used to explain why ferrous ions inhibited the accumulation of riboflavin by *Clostridia*, bacteria which form hydrogen peroxide. The inhibition was viewed as destruction of the vitamin rather than failure of synthesis. Fluorescence microscopy has continued to attract attention as a means of localizing riboflavin and thiamine in cells (36).

A few studies were made during 1946 of the physiological effects of riboflavin. Bowles *et al.* (32) compared the ocular lesions of ariboflavinotic rats with those seen in vitamin A deficiency and reemphasized the conclusion that corneal vascularity is not a specific sign of lack of riboflavin. The responses of deficient rats to reduced barometric pressure were investigated by Wickson & Morgan (33) who found that riboflavin corrected the physiological disturbances in carbohydrate metabolism brought about by this additional strain on the depleted animals. The production of an x-ray induced mutant of *Neurospora* which required riboflavin for growth might have been considered routine had it not been for the fact that the occurrence of the vitamin requirement was conditioned by the temperature at which growth took place (34). Below 25°, riboflavin was not essential, whereas above that temperature it was. A similar dependence of growth factor requirement on temperature of incubation had been observed by Robbins & Kavanagh (35) with *Phycomyces* in its need for thiamine and for hypoxanthine.

## Nicotinic Acid

*The etiology of pellagra.*—When nicotinic acid was shown to cure pellagra an easy explanation for the causation of the disease was provided. Pellagra could be said to result from a deficiency of nicotinic acid in the diet, and the long-established relationship between the incidence of the malady and the eating of corn could be attributed to the marginal amount of nicotinic acid in this cereal. However, when quantitative methods for the estimation of the

vitamin in foods were developed, this explanation of the role of corn in the etiology of pellagra was questioned because it was found that an adequate amount of the vitamin could be obtained from a diet composed largely of corn. For example, Aykroyd & Swaminathan (37) observed that a diet based on corn, and which was pellagragenic, provided about 15 mg. per day of nicotinic acid, while certain nonpellagragenic regimens which did not include corn contained only about 5 mg. of the vitamin in a day's intake. This was a clear indication that even though there was no superfluity of nicotinic acid in corn, its role in the etiology of pellagra was not simply its lack of this vitamin. Krehl *et al.* have demonstrated in a series of publications that corn will induce a need for nicotinic acid in rats, a species which can synthesize the vitamin, and that tryptophane as well as nicotinic acid will meet this need (38, 39, 40, 43). They have felt that postulated influences on the intestinal flora of the rats and imbalances of amino acids are largely responsible for the deleterious action of corn. They showed (38) that a need for nicotinic acid could be induced in the rat not only by the feeding of corn in a low protein diet, but also by the addition of gelatin or of acid hydrolysates of proteins (free of tryptophane) to a low protein ration. Briggs (41) has reported similar observations with chicks. In fact, Groschke & Briggs have attributed the pellagragenic action of gelatin in the chick to its high content of glycine and its lack of tryptophane (42). Niven *et al.* (44) likewise have reported deleterious effects from tyrosine and phenylalanine added to low protein diets such as those used by Krehl *et al.* In this instance, however, relatively large amounts of nicotinic acid were required to counteract the toxic manifestations. With pigs Wintrobe *et al.* (45) found that rations containing 26 per cent casein were adequate for growth without added nicotinic acid, whereas those with less protein caused the appearance of deficiency signs unless nicotinic acid was present. Similarly, Cooperman *et al.* (46) attributed the previously reported production of a pellagrous syndrome in monkeys (47) to the fact that the rations were low in protein. With high protein diets the vitamin deficiency was not produced. It must be clear from these studies that the amount of tryptophane in the ration in relation to the intake of other amino acids is of importance in the etiology of pellagra. Some of the proteins of corn are known to be deficient in tryptophane.

The ability of both trytophane and nicotinic acid to overcome

the deleterious effects of corn suggested to Rosen *et al.* (48) that tryptophane was the precursor used by the rat for the synthesis of nicotinic acid. In support of this hypothesis they demonstrated that administration of the amino acid led promptly to greatly increased excretion of the vitamin. Krehl *et al.* (40) earlier had viewed the action of nicotinic acid as improving tryptophane utilization and that of tryptophane as stimulating nicotinic acid synthesis. However, the observations of Rosen *et al.* are most readily explained on the basis that nicotinic acid arises from tryptophane.

Another aspect of the etiology of pellagra has been the demonstration by Woolley (49) of a pellagragenic agent in corn which will bring about in mice fed a low protein diet, a disease similar to pellagra. This toxic substance was concentrated about 100,000 times so that approximately 1 mg. per 100 gm. of ration was sufficient to cause disease. It appeared to be an organic base, and it was postulated that the substance might be an inhibitory structural analogue of nicotinic acid. Woolley had shown previously that a pellagra-like disease of mice could be produced by feeding a structural analogue of the vitamin, namely 3-acetylpyridine (50) and that it could be prevented either with nicotinic acid or with tryptophane (51). Following this finding of a pellagragenic substance in corn, Kodicek *et al.* (52) showed that indole acetic acid in doses comparable to that of Woolley's concentrate from corn retarded the growth of rats in a manner reversible by either nicotinic acid or tryptophane. Although indole acetic acid per se was probably not in the concentrate described by Woolley, Kodicek *et al.* pointed out that sufficient indole acetic acid, free and combined, is known to exist in corn to produce a pellagragenic effect. The structural analogy of indole acetic acid to both nicotinic acid and to trytophane is evident.

In the light of all these studies the etiology of pellagra appears to be not merely a matter of deficiency of nicotinic acid in the diet. The association of the eating of corn with the occurrence of the disease is classical. If the pellagragenic agent did not exist in corn it is probable that pellagra would be rare. If corn were richer in tryptophane it is likely that the disease would be rare. If corn contained more nicotinic acid the effects of these other factors might be submerged, and again the incidence of the malady might be greatly reduced. The relationship of the disease to protein intake, and to postulated toxic agents in corn has been discussed for

almost a century, but these aspects of the etiology lost favor in the face of the vitamin hypothesis. It is interesting to see how all three aspects have gained more solid experimental backing.

*Biosynthesis of nicotinic acid.*—An elegant investigation of Bonner & Beadle (53) has led to the isolation of the immediate precursor in the biosynthesis of nicotinic acid. Three x-ray induced mutants of a *Neurospora* were produced which required nicotinic acid for growth, i.e., they were unable to synthesize the vitamin. The three were not, however, identical genetically, and hence it was postulated that each was unable to perform one of a consecutive series of reactions leading to the formation of nicotinic acid. By using the mutant unable to carry out the synthesis most remote from nicotinic acid as the tool for assay, and the one unable to achieve the synthesis next to the vitamin as a means of producing the precursor, this latter substance was isolated and shown to be a pyridine derivative containing seven carbon atoms. The last step in the biosynthesis of nicotinic acid would thus appear to be a degradation of this more complex compound. Similar work leading to the isolation of the two other precursors, two steps and three steps removed from nicotinic acid, will be awaited with much interest. In view of the findings cited in the previous section, tryptophane was tested to learn whether it was one of these precursors, but it did not prove to be so. If tryptophane is a precursor of nicotinic acid in *Neurospora*, therefore, it must be at least three enzymic steps removed from the vitamin.

*Other studies.*—Just as nicotinic acid appears to be formed by the degradation of a larger molecule, so it is metabolized further by conversion to more complex products. A fraction of ingested nicotinamide is methylated by some species and excreted as $N_1$-methylnicotinamide. A second metabolic product from the vitamin has now been demonstrated by Knox & Grossman (54) who showed that about 30 per cent of ingested nicotinamide is found in the urine as the 6-pyridone of $N_1$-methylnicotinamide. They were led to this discovery by searching for the possible normal substrate of an enzyme in liver which oxidized quinine. $N_1$-methylnicotinamide appeared to be this substrate. Roggen (55) has detected a substance in urine which yielded nicotinic acid when oxidized. In the light of the work of Bonner & Beadle (53) one cannot but wonder whether this material may not be related metabolically to nicotinic acid.

Although the microbiological and the colorimetric (cyanogen bromide) methods of estimating nicotinic acid seem well entrenched, Scudi (56) has proposed a highly sensitive and more accurate fluorimetric modification of the latter procedure.

Additional studies have been reported on the manifestations of nicotinic acid deficiency in animals. For example, Briggs (57) observed that perosis could result in turkey poults due to lack of this vitamin. This finding serves to emphasize the nonspecific nature of several signs of deficiency diseases. Thus, perosis in birds may result not only from lack of choline or biotin or folic acid or nicotinic acid, but also from a manganese deficiency. It is likewise well known that anemia is scarcely more specific than failure of growth, since so many deficiencies can cause it. Hegsted (61) has found that ducklings readily develop signs of nicotinic acid deficiency. In attempting to produce aniacinosis in monkeys Cooperman *et al.* (46) found that with semipurified diets free of the vitamin a disease was induced which did not respond to nicotinic acid administration. Since liver was curative of this malady, and since a response of monkeys to nicotinic acid had been demonstrated previously(47) a multiple deficiency may have complicated these studies. On the other hand, the high casein content of the purified diet may have obviated the need for nicotinic acid. An example of the interrelationship between dietary essentials was recorded by Raoul (58) who found that riboflavin deficiency of the rat led to a reduced amount of nicotinic acid in the liver much as thiamine deficiency is known to influence the riboflavin content of this organ (59), or folic acid deficiency is said to reduce hepatic pantothenic acid (60), and athiaminosis to lower hepatic ascorbic acid (199). Likewise Lecoq (62) reported signs of nicotinic acid deficiency in rats lacking simultaneously nicotinic acid, pantothenic acid and *p*-aminobenzoic acid.

## Pyridoxine

The year 1946 has seen investigations of pyridoxine influenced in considerable measure by the notable discovery of the preceding year that pyridoxal phosphate was the coenzyme of amino acid decarboxylases. Thus Lichstein *et al.* (63) have continued their studies and shown that pyridoxal phosphate is a part of the transaminase system and of the tryptophane-synthesizing mechanism of *Neurospora* (64). Lyman *et al.* (65) have shown that carbon dioxide and pyridoxine reduce qualitatively the amino acid re-

quirements of lactic acid bacteria. This they have interpreted to mean that the pyridoxine series of compounds functions in the synthesis of amino acids through carboxylation, the reverse of the reaction already known to depend on this vitamin. Stokes & Gunness (66) somewhat earlier had also observed that pyridoxine eliminated the need of bacteria for certain amino acids. Another metabolic function for the vitamin has been suggested by the claim that oxidation of fatty acids by kidney slices is accelerated by *in vitro* addition of pyridoxine (67).

A number of studies has been made of the manifestations of pyridoxine deficiency in animals. One outcome of these investigations has been the realization that rations in practical use may be marginal with respect to this vitamin. Bird & Rubin (68) found that natural rations free of animal protein led to incipient pyridoxine deficiency in chickens. The Red Rock breed was shown to have an unusually high requirement for the vitamin (69). The quantitative requirements of adult chickens for pyridoxine have been determined (70). Another aspect of this marginal supply of pyridoxine in grains has been the production of a "conditioned" deficiency disease by the addition of large amounts of thiamine to wheat flour (71). When this fortified material was given to rats a malady was induced which was similar to that seen in pyridoxine deprivation, and which was preventable by the vitamin.

The signs of pyridoxine deficiency in various animal species have many similarities, and therefore the study of monkeys deprived of this vitamin (72) has served to annotate this generalization, and to add to our knowledge of the manifestations. A new sign of the disease has, however, been found by Stoerk & Eisen (73) who showed that in rats the deficiency state was accompanied by a marked reduction in ability to form circulating antibodies to sheep erythrocytes. This condition was attributed to atrophy of the thymus in the depleted animals (74). In this latter study use was made of desoxypyridoxine (2,4-dimethyl-3-hydroxy-5-methylolpyridine), an analogue of the vitamin which Ott (75) had demonstrated to be a powerful antagonist to it in the chick. When desoxypyridoxine was fed, a disease was elicited which could be prevented by increasing the pyridoxine content of the ration. Similarly this inhibitory structural analogue caused atrophy of the thymus in rats, and an attendant reduction in circulating antibodies.

## Pantothenic Acid

Most of the studies with pantothenic acid during 1946 were concerned with signs of deficiency of the vitamin. Thus, Nelson & Evans (76) found that adult female rats failed to reproduce when this vitamin was withheld from the diet. Resorption of the embryos was a common finding. This observation was significant because of the rapidity with which the abnormality followed the dietary restriction. In adult animals it is well known that the signs of a vitamin deficiency, in contrast to the situation in growing individuals, develop only after prolonged deprivation (77). Here, however, resorptive failure of pregnancy could be noted when the pantothenic acid free ration was begun on the day of mating. In addition to the recognized signs of pantothenic acid deficiency in rats, Jürgens & Pfaltz (78) found that congestion or inflammation of the respiratory tract frequently was produced. It has been known for several years that the porphyrin caked whiskers observed in pantothenic acid deficient rats may also be produced by withholding water from normal animals. Perhaps the finding that pantothenic acid deficient rats are more subject to water intoxication (79) may be related to this situation. In any event, the effect of the vitamin on water tolerance was viewed in relation to adrenal function since it is known that in rats, the lack of pantothenic acid frequently results in adrenal hemorrhage. The production of signs of pantothenic acid deficiency in monkeys fed a highly purified diet has been described (72). Many of the manifestations were similar to those previously noted in other species.

Investigations have been continued to learn the role of pantothenic acid in human physiology, but no clear picture has emerged. From studies on normal subjects the previously indicated conclusion that injected vitamin can be recovered to only a limited extent in the urine has been confirmed (80, 81, 82). It is likewise clear that the lactone moiety of pantothenic acid does not account for the remainder of an injected dose. The renal clearance of the vitamin has been determined (83).

As with nicotinic acid, Nelson & Evans (84) observed that increasing the casein content of a diet practically eliminated the need for pantothenic acid. However, no single amino acid was implicated as tryptophane has been in the case of nicotinic acid.

## Biotin

Tatum (85) has published evidence for the pathway of biosynthesis of biotin in fungi. By use of selected species it was shown that some forms which cannot synthesize the vitamin nevertheless, build up considerable concentrations of desthiobiotin in the medium. Taking into account the previously acquired bits of evidence, it was postulated that the route of biotin synthesis is through pimelic acid to desthiobiotin and finally to the vitamin. The last step, namely the insertion of a sulfur atom into desthiobiotin has been demonstrated to occur in yeast (86).

More work has been done on oxybiotin, the analogue of biotin in which the sulfur atom has been replaced by an oxygen, and it is now clear that this substance has vitamin activity for a variety of living things, including animals, (87, 88, 89) as well as bacteria, and that such activity is not due to a biological conversion of the analogue to the vitamin. Oxybiotin can therefore substitute for biotin just as 2-methylnaphthoquinone can replace naturally occurring vitamin K. Another less readily understandable substitution for biotin is that found by Williams & Fieger (90). These investigators showed that for *Lactobacillus casei* the inclusion of oleic acid in the medium allowed good growth to occur in the absence of biotin. In the presence of the fatty acid no biotin synthesis could be demonstrated.

## Inositol

Posternack (91) has continued his basic work on the chemical configuration of the inositols, and has investigated the structure and configuration of mytilitol, a methyl inositol found in mussels.

Several papers have been published to show that in a variety of situations either biotin or inositol may provide the same vitamin effect. In all instances, biotin was qualitatively and quantitively slightly better. Several years ago Pavcek & Baum (92) reported that the spectacled eye condition of rats reared on a synthetic type of diet was cured by administration of inositol. Shortly thereafter Nielsen & Elvehjem (93) stated that biotin was the curative agent for this sign in rats. Now, Lindley & Cunha (94) have observed that when pigs are fed a highly purified ration plus sulfaphthallidine they developed a syndrome similar to that seen in egg-white-induced biotin deficiency of these animals (95). This disease could be combatted either with biotin or with inositol. Furthermore,

Spitzer & Phillips (96) have stated that when the protein of an otherwise synthetic diet for rats was supplied as soybean oil meal, an alopecia identical in type to that seen in inositol deficiency of mice developed. This condition was prevented either by inositol or by biotin. This interchangeability of the two vitamins is obviously a situation requiring more study. Best *et al.* (97) have examined the lipotropic actions of inositol and of choline and have denied the statement of Gavin & McHenry (98) that biotin has a fatty liver producing effect which is counteracted by inositol.

By use of the isotope technique, Stetten & Stetten (99) have concluded that glucose can arise from inositol in the rat. Since the positive evidence rested on a somewhat equivocal experiment with one rat the need for confirmation should be considered.

## Choline

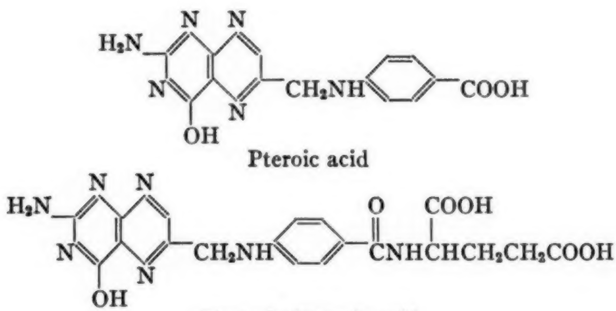
The precursors in the biosynthesis of choline have been established by a series of studies in several laboratories. Perhaps the most direct demonstration has been that of Horowitz (100,101) who used a series of genetically dissimilar mutants of a *Neurospora*, each one postulated to lack the ability to carry out one step in the synthesis of choline. The working hypothesis and mode of attack were the same as those used subsequently in the same laboratory for the elucidation of the biosynthesis of nicotinic acid. By growing the mutant strain which was unable to complete the last stage in the synthesis and depending on it to accumulate the immediate precursor of choline in the medium, this substance was produced and isolated. Assays for it were done with a mutant strain which failed to achieve an earlier step in the synthesis, but which could convert the immediate precursor to choline. The substance proved to be dimethylethanolamine. As a result of the investigations of Horowitz with *Neurospora*, and of Jukes *et al.* (102) with chicks and *Neurospora* it is clear that ethanolamine is methylated stepwise to yield the monomethyl, then the dimethyl derivative, and finally the trimethyl compound (or choline). Jukes *et al.* (102) have pointed out that dimethylaminoethanol has long been known as a constituent of some legumes. The experiments with chicks, indicated that a substance, not choline, but possessing biological activity, was present in foods. Lucas *et al.* (103) have recently presented evidence of a similar nature. Du Vigneaud *et al.* (104) have continued their use of isotopic tags to study this problem of the

synthesis of choline, and have concurred in the belief that it progresses as outlined above. They assigned a prominent role to dimethylaminoethanol because of their finding that it not only served as an acceptor of methyl groups, but also as a donor of them.

To those interested in the etiology of cancer the report of Copeland & Salmon (105) will be noteworthy. These investigators described the appearance of apparently malignant changes in the livers of rats maintained for long periods on choline deficient rations.

## Folic acid

At last, the isolation and determination of structure, and synthesis of folic acid have been described (106). Coupled with its effective use in the treatment of various human anemias, and flanked by numerous studies of its action on animals, this is the biggest story of 1946 in the field of water-soluble vitamins. Four



Pteroic acid

Pteroyl glutamic acid

Fig. 1.—Structures of Pteroic Acid and Pteroyl Glutamic Acid.

chemically and biologically distinct substances with folic acid activity are known to occur naturally. The simplest, the *Streptococcus lactis* R factor (SLR factor) may well be pteroic acid (Figure 1) although this has not been finally established. The second compound, which is the one isolated from liver, has been shown to be pteroyl glutamic acid. This was established by degradation as well as by synthesis (106). Pteroyl glutamic acid is the only synthetic folic acid thus far available for biological experimentation. It is identical with vitamin $B_c$. The third compound is pteroyl triglutamic acid, which is isolated from the fermentation products of a

diphtheroid bacterium. Because of the greater ease of isolation of this compound it was the one on which most of the work leading to the determination of structures of folic acids was done. It has not yet been synthesized, although it has been degraded to pteroyl glutamic acid. The fourth compound is pteroyl heptaglutamic acid (107) and is the vitamin $B_c$ conjugate isolated from yeast in crystalline form. It has not been synthesized, but its conversion to pteroyl glutamic acid by an enzyme from animal tissues was described previously (108, 109). The isolation of a peptide containing *p*-aminobenzoic acid and a number of glutamic acid residues from yeast has been described by Ratner *et al.* (110). Although this substance has no recognizable vitamin action it may have arisen by autolytic degradation of a folic acid compound. The work which led to the isolation, determination of structure, and synthesis of liver folic acid (pteroyl glutamic acid) and of the conjugates was conducted by a large group of investigators at the Lederle Laboratories (106) and by a group at Parke, Davis & Co. (107).

The determination of structure of pteroyl glutamic acid and of the triglutamic acid depended on the alkaline oxidative cleavage to 2-amino-4-hydroxy-6-carboxypteridine; 2-amino-4-hydroxy-6-methylpteridine; *p*-aminobenzoic acid; and glutamic acid. Some of these constituents were first recognized as products of acid hydrolysis. The synthesis of pteroyl glutamic acid was accomplished by a one-step coupling of 2, 4, 5-triamino-6-hydroxypyrimidine with *p*-aminobenzoyl glutamic acid and dibromopropionaldehyde.

Numerous investigators have examined the therapeutic effects of synthetic pteroyl glutamic acid on various macrocytic anemias of man (111 to 116). In summary it can be said that all agree that partial or even complete remission of the hematological changes may be produced in pernicious anemia and in sprue and in macrocytic nutritional anemia. Claims have been made that some anemias of pregnancy likewise are benefited. Despite the unquestioned influence of this vitamin on pernicious anemia, it is clear that the antipernicious anemia factor of liver is not folic acid because concentrates of the latter contain practically no folic acid (117), and because of certain qualitative differences between the response elicited by the vitamin and by the liver principle. The existence in foods of conjugates of pteroyl glutamic acid and the occurrence in animal tissues of special enzymes which hydrolyze these have led

some investigators to explain the anomaly in terms of these conjugates and enzymes (118). Indeed, it is reported that the normal man differs from the pernicious anemic in his ability to metabolize pteroyl heptaglutamic acid (118). However, it seems too early to give any adequate explanation of why both folic acid and a vitamin-free liver extract cause remission of the disease. The possession of specific vitamin activity by compounds structurally quite dissimilar to the vitamin concerned has already been noted in this review in the sections on thiamine and inositol. Observations pertinent to the role of folic acid in pernicious anemia were made by Jacobson & Simpson (119) prior to the elucidation of the structure of the vitamin. They observed that the pterin content of cells in the mucosa of the alimentary tract which are known to be altered in pernicious anemia, was very much changed in the disease.

A large number of reports have increased our knowledge of the signs of folic acid deficiency in diverse species. Much of this work has employed the chick as test object because this species readily develops signs of deficiency when folic acid is withheld from the diet. With rats experimental tricks such as the administration of drugs are necessary to produce a dietary need for the vitamin. The quantities of folic acid necessary for maximal response in growth or hematological changes have been determined (120 to 125, 167), and it has been confirmed that the conjugates are as effective on a molecular basis as pteroyl glutamic acid (126). The need for vitamins $B_{10}$ and $B_{11}$ seems to have disappeared when adequate amounts of folic acid were included in the basal ration (125, 140), because chickens may be reared to maturity and through a second generation on a diet in which all the water-soluble vitamin supplements are of known constitution (141). The existence of these two postulated vitamins was never established by the use of a basal ration lacking only one of them, and adequate with respect to all other vitamins, but rather depended in part on a differential assay of the ability of a given concentrate to promote growth (vitamin $B_{11}$), or to foster good feather production (vitamin $B_{10}$).

Lewisohn *et al.* (127) have reported that pteroyl triglutamic acid arrested the growth of transplanted mammary tumors of mice when it was injected intravenously. Pteroyl glutamic acid did not do this and, indeed, prevented the tumor inhibiting action of the conjugate. This group of workers also reported a similar antagonism of the triglutamic acid derivative with xanthopterin (128).

A response, usually hematological, may be obtained to folic acid in rats placed under stress by administration of drugs such as the sulfonamides (129); or by protein depletion or deprivation with respect to riboflavin (130) or pantothenic acid. Likewise, dogs fed a folic acid free basal diet, and repeatedly subjected to nicotinic acid deficiency, become refractory to treatment with the latter vitamin unless folic acid is administered to them (131). More reports of the effects of folic acid deficiency in monkeys and in mink have appeared (132 to 136).

The elucidation of the structure of folic acid, and especially the finding that *p*-aminobenzoic acid is a part of the molecule, has pointed the way to an understanding of the mode of action of sulfanilamide and its derivatives. If it may be assumed that *p*-aminobenzoic acid is a substrate from which pteroyl glutamic acid is synthesized in nature, then the structurally similar sulfonamides would be expected to compete with it in this reaction. The substrate would be *p*-aminobenzoic acid, and the product of the reaction would be folic acid. Now Miller demonstrated several years ago (137) that sulfonamides markedly inhibited the formation of folic acid by bacteria. Furthermore, Lampen & Jones (138) have found that those bacteria which require folic acid, and therefore cannot synthesize it, are not susceptible to inhibition by sulfonamides. Presumably, these species have no synthesizing enzyme system to be inhibited. In addition, Lampen & Jones observed that in some bacterial forms, pteroyl glutamic acid would erase the bacteriostatic effect of the sulfonamides just as well as did *p*-aminobenzoic acid, but whereas the antagonism between the drugs and the latter metabolite was competitive in nature, that between the sulfonamides and folic acid was not. In other words, the same amount of the vitamin overcame a large dose of the drug equally as well as it did a small one. This would be expected if folic acid were the product, and *p*-aminobenzoic acid the reactant or substrate, of a reaction subject to competitive inhibition. Now, the animal being limited or completely lacking in ability to synthesize folic acid is not subject to sulfonamide inhibition (cf. the folic acid-requiring bacteria) while the parasite which can make folic acid, is vulnerable to attack by the drugs. While this may not be the final explanation of sulfonamide action, at least it is a long step ahead. Since the pteridine ring system is a derivative of pyrazine, the findings of Stetten & Fox (139) that in the presence of sulfonamides bacteria accumulate a pyrazine compound in the medium is of in-

terest.[2] Perhaps this abnormal metabolite is merely a stage in the synthesis of folic acid, and builds up in the medium when the synthesis is blocked.

### Strepogenin

Since this new growth factor has not previously been treated in the section on water-soluble vitamins, a brief review of its history may be in order. It was discovered by Woolley in 1941 (142) as a nutritive essential for certain hemolytic streptococci. The properties which were found as a result of numerous attempts to isolate it indicated that it was of peptide nature (142, 143, 144) and led Sprince & Woolley (144) to examine protein digests for it and to find that crystalline proteins were by far the richest natural sources. Pollack & Lindner (145) had observed a growth stimulant for *Lactobacillus casei* to be present in peptone, and although Chu & Williams (146) had concluded that it was *p*-aminobenzoic acid plus glutamine plus pyridoxine, Sprince & Woolley demonstrated that it was very similar to, if not identical with their streptococcal growth factor (143). Simultaneously, these authors, and Wright & Skeggs showed that the *L. casei* stimulant was present in digests of casein (147). Since it was known that mixtures of amino acids were inferior to intact protein for promotion of growth of animals, and since Sprince & Woolley (144) had demonstrated the existence of a new bacterial growth factor in some proteins, it seemed possible that this substance, strepogenin, might be the part of some proteins necessary for maximal growth of animals. Woolley (148, 149) showed that all proteins were not able to bring about this growth effect in mice fed amino acids as a nitrogen source, and that the potency of a protein in this regard correlated nicely with its strepogenin content as determined microbiologically. Furthermore, intact egg white, a protein adequate from the standpoint of amino acid composition, but deficient in strepogenin, could be used as a source of nitrogen in the basal ration on which to demonstrate the action of proteins such as casein and trypsinogen, which are rich in the growth factor. Concentrates of strepogenin prepared from tryptic digests of casein were equal in effect to casein when fed on an equivalent strepogenin level. Womack & Rose (150) were able

[2] Since this was written, Shive et al. (200) have identified this substance as amino-imidazolecarboxamide and have regarded it as the immediate precursor of purines.

to confirm the existence in casein of a growth factor for rats, and reported that it had properties in common with strepogenin. It seems clear that some proteins contain a nutritional factor of importance to many species, and that the old conclusion that the nutritive powers of proteins are measured only by amino acid content needs revision.

Strepogenin has not yet been obtained in pure condition, but available evidence indicates that it is a rather small peptide of acidic character (144, 151). The evidence for its peptide nature has been summarized (151), and it has been shown that the synthetic peptide seryl glycyl glutamic acid has strepogenin activity, although not of sufficient magnitude to justify the conclusion that it is identical with the natural substance (152). Totter & King (153) have reported that strepogenin concentrates markedly influence the response of some bacteria to glutamic acid.

## B Complex

Several studies have been made which involve a number of the water-soluble vitamins rather than one specific member of the group. For example, the measurement of the amounts of the various vitamins in natural products such as colostrum and whole milk (154), canned foods (155), and in foods served in restaurants (156) have continued to attract attention. Pathological alterations brought about by B complex deficiency such as kidney hypertrophy likewise have continued to excite notice (163). A series of experiments by Lecoq *et al.* (157 to 160) has revealed that in total B complex deficiency chronaxia is altered. Although lack of thiamine, or riboflavin, or nicotinic acid produces this effect, the major part of it is ascribable to pantothenic acid deficiency. Possibly this is related to the well-known alteration in nerves which results from deprivation of this vitamin.

Several studies have been made of the B-vitamin needs of insects. These investigations are continuations of those begun in previous years, and have shown requirements for insects quite similar to those of higher animals or of lower plants (microorganisms). Even the clothes moth it seems cannot get along without its vitamins (161) which it presumably gets from perspiration or food spilled on the cloth. Work with certain insects which normally harbor microbial symbiants in a special organ has shown quite clearly that when these species are reared from aseptic eggs their

vitamin requirements become more numerous (162). This then is the first direct experimental evidence for the much discussed hypothesis that intestinal microorganisms supply the host with nutritive essentials.

## New and Unidentified Vitamins

Several previously unrecognized nutritive factors have been described during 1946. Rubin & Bird (164, 165, 166) observed that although chicks would grow quite well on a highly purified diet containing casein, they did less well when vegetable protein such as that of soybean replaced the casein, even though supplies of the recognized vitamins were adequate. A growth stimulant was recognized in cow manure and in the feces of normal chickens, and from the former source it was concentrated to a high degree by these workers. It was a nondialyzable substance. Possibly this growth factor may be related to strepogenin, although there are no data bearing on this point.

The use of vegetable protein in place of casein in simplified diets for rats likewise led Jaffé (168) to the recognition of a new growth factor. Liver extract was found to contain this substance, and it was thought not to be related to strepogenin because it was destroyed by heating to 100°. Furthermore, Cary *et al.* (169) discovered that exhaustive extraction of casein with hot alcohol rendered it inadequate for normal growth of rats unless a concentrate prepared from liver was also added. Twenty μg. per day of this latter substance was adequate.

In addition to the above, heat-labile nutritive essentials have been recognized by the use of other species. It is not clear whether the same substance is responsible for all the observed effects or whether there are a multiplicity of thermolabile factors. Cooperman *et al.* (170, 171) found that monkeys on a highly purified ration made deficient with respect to folic acid or to riboflavin developed an anemia when the missing vitamin was provided. The disease could be prevented or cured only by the inclusion of fresh liver or raw milk. Heating of such foods resulted in a loss of potency. The factor involved was called the monkey antianemia factor. Wilson *et al.* (132) in their study of the antianemic properties of folic acid in monkeys may have encountered a deficiency of this new agent because they too observed that the hematological

response to administered folic acid was highly variable. Apparently the production and subsequent cure of a deficiency of folic acid or riboflavin is a prerequisite to the establishment of need for the monkey antianemia factor. Cooperman *et al.* (172) also recognized a heat-labile component of liver which stimulated early growth of *Streptococcus faecalis*, and speculated about its relationship to the monkey antianemia factor.

Watson & Castle (173) have called attention to the fact that crude liver extracts are far more active in the treatment of certain human anemias of pregnancy than are purified concentrates of the antipernicious anemia factor, and hence have indicated the presence of another hematologically active substance in liver. Whether or not it is folic acid was not established. Rossi (174) and Mazza *et al.* (175) have made a study of means of concentrating the extrinsic or dietary factor in pernicious anemia, but have not succeeded in obtaining it in pure form.

Although highly purified casein-containing diets are known to be adequate for the growth of rats, it is also known that they are not quite the equal of a good stock ration. This has been underscored again by the findings of Richardson & Hogan (178) that hydrocephalus was present in an appreciable number of animals born from mothers raised on highly purified diets. Another example is the observation of Nelson *et al.* (179) that rats maintained on a highly purified diet developed leucopenia and granulocytopenia during the reproductive cycle, whereas stock fed rats did not. The manifestations in this latter case, however, are suggestive of a need for folic acid.

Finally, a nutritive essential for *Lactobacillus gayonii* has been described (176) which appears to be one of the nucleotides obtainable from nucleic acid (177).

Although the number of unknown vitamins would appear to be large from the foregoing discussion, experience has shown that frequently several postulated nutritive essentials disappear after a new factor has become available in pure forms. Therefore, it may be that the need for some of the unknowns described in this section will be met adequately by folic acid and by strepogenin. It is likewise probable that some of these unknowns will become established as new dietary essentials.

## Antagonistic Structural Analogues of Vitamins

One of the important advances in the study of vitamins during the past few years has been the discovery that compounds closely related in structure to each of the several vitamins may be capable of producing the characteristic signs of deficiency which then may be erased by increasing the dietary intake of the vitamin in question. The findings in this regard and the interpretation of these as aids to the understanding of metabolic processes have been reviewed by several authors during 1946 (180 to 185). It is probable that these studies will be of considerable importance in the elucidation of the ways in which vitamins function. An illustration of this was discussed above under folic acid.

Space does not permit a full treatment of findings in this section of vitaminology, especially since the reviews mentioned in the preceding paragraph are readily available. Suffice it to say that during the period reviewed more antagonistic analogues of the vitamins were described and frequently used to study biochemical reactions. Thus, araboflavin was found to antagonize the action of riboflavin (186) and desoxypyridoxine that of pyridoxine (75). More pantothenic acid inhibitors were discovered (187, 188, 189). McIlwain (190, 191) has used one inhibitory structural analogue of pantothenic acid as a tool with which to demonstrate that bacterial cells build pantothenic acid into a more complex substance which presumably then functions in metabolism. The work of Neal & Strong (192) had already demonstrated that an alkali stable conjugate of this vitamin occurred in liver, and possibly the pantothenic acid analogue in the work of McIlwain inhibited the synthesis of this conjugate from the vitamin. Woolley (193) has reported that another analogue of pantothenic acid, phenyl pantothenone, was antagonized in its bacteriostatic action not only by the vitamin but also by glutamic acid, proline or histidine. This same analogue has been found efficacious in the treatment of blood-induced malaria of birds and man (182, 194). Triethyl choline has been found to compete with choline in some of its pharmacological effects (195). Kirkwood & Phillips (196) have found that the new insecticide, hexachlorocyclohexane, which is an analogue of inositol, actually competes with that vitamin acting as a growth factor for yeast. In view of this it would be of interest to determine whether its action on insects might also be antagonized by the vitamin.

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# THE USE OF PTEROYLGLUTAMIC ACID (LIVER *L. CASEI* FACTOR, FOLIC ACID) IN CLINICAL STUDIES[1]

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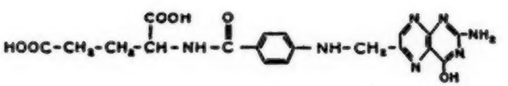
The rise of experimental medicine has been characterized by the physician's ability to comprehend some of the secrets of bodily derangement. Each major step toward an understanding of the mechanism of a specific derangement has brought a change in the physician's approach to the problem. No matter how dramatic the step is, however, the true clinical investigator is not satisfied with the progress made. It is the progress still to be made that challenges him. The enterprising hematologist and nutritionist have long dreamed of unlocking the fundamental secrets of blood formation. Despite the fact that for a number of years there was little advancement, the quest has continued in many laboratories and clinics. Now, through the convergent researches of many investigators, a basic chemical molecule of great significance has been found and gives promise of being a key to some of these secrets. As a result, investigators in the fields of nutrition and hematology are at the threshold of a future greater than they can foresee. Moreover, a new therapeutic approach is afforded the physician. He can now use a synthetic chemical substance to relieve persons with sprue, pernicious anemia and related anemias.

This new therapeutic substance is sometimes called "folic acid." It is the youngest member of the vitamin family. Folic acid was the name given to a material obtained in nearly pure form from spinach by Mitchell *et al.* (1). Strictly speaking, the liver *L. casei* factor should not be called folic acid. The term folic acid was given to a substance which supports growth of two organisms commonly used in microbiological investigation: *Lactobacillus casei* and *Streptococcus lactis R* (now termed *S. faecalis*). Its potency for *S. lactis R* was used as a guide in purification procedures, and it is defined specifically as a growth factor for that organism. It seems almost certain that at least four crystalline forms of "folic acid" have been obtained from liver, yeast, and

[1] This review covers the period from September, 1945 to September, 1946.

other sources. Their chemical structures are becoming known, and their relationship, one to the other, will soon be clarified. Liver *L. casei* factor has been synthesized recently, and its structure as pteroylglutamic acid (Fig. 1) has been established (2). The compound obtained from the fermentation residue differs from the

LIVER L. CASEI FACTOR



N-[4-{[(2-amino-4-hydroxy-6-pteridyl)methyl]amino}benzoyl] glutamic acid

FIG. 1

above in that it contains two more molecules of glutamic acid than the liver *L. casei* factor. The vitamin $B_c$ conjugate (Fig. 2) has six more molecules of glutamic acid than has pteroylglutamic acid, making seven in all.

About a year ago the synthetic liver *L. casei* factor was first administered to human beings. Berry *et al.* (3) in their studies of nutritional deficiencies accompanied by leukopenia found that either the liver *L. casei* factor or a folic acid concentrate would elevate the total number of circulating leukocytes. There was a proportionate number of granulocytes and a left shift in the Arneth nuclear index which was interpreted as reflecting a direct bone marrow delivery.

Spies *et al.* (4) selected a number of cases of macrocytic anemia for treatment with niacin, thiamine, riboflavin, calcium pantothenate, pyridoxine, inositol, *p*-aminobenzoic acid, choline, pyridoxamine, and pyridoxal. The diets were rigidly controlled so that no meat or meat products were ingested. These substances were found to have little or no efficacy in producing hematological regeneration. Under the same dietary controls, synthetic liver *L. casei* factor was administered parenterally to five cases of macrocytic anemia in relapse. The material was dissolved in saline solution made slightly alkaline with small amounts of sodium bicarbonate. To an additional four cases liver *L. casei* factor was given orally. Reticulocytosis occurred promptly, and the red blood cell counts and hemoglobin content rose in every instance.

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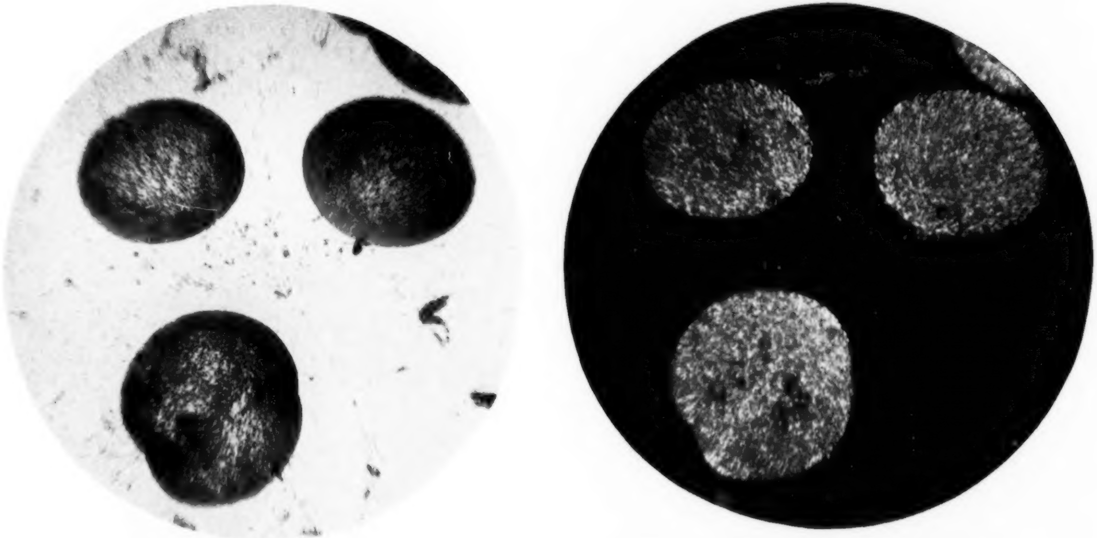

FIG. 2.—Photomicrographs of crystalline vitamin $B_c$ conjugate (pteroylhexaglutamylglutamic acid) isolated from yeast; taken in plain light and with crossed nicols (×125). From an article by J. J. Pfiffner, D. G. Calkins, E. S. Bloom, & B. L. O'Dell, (In preparation).

Vilter *et al.* (5) next studied fourteen cases of macrocytic anemia. Five of these were diagnosed as Addisonian pernicious anemia, six were nutritional macrocytic anemia, and three were of indeterminate types. All but two of the patients were hospitalized, and their diets were restricted to exclude meat and meat products. Protein intake was minimal so that any response could be attributed to the liver *L. casei* alone. The possibility of any so-called spontaneous remission was also thereby diminished. The long clinical association between achylia gastrica and Addisonian pernicious anemia, and the frequent absence of achylia gastrica in the other related anemias make it of sufficient interest to print the following table (Table I) concerning the series of patients studied.

Thirteen of the fourteen patients had a positive hematological

TABLE I

STATUS OF GASTRIC CONTENTS AFTER HISTAMINE PHOSPHATE STIMULATION

| | |
|---|---|
| Case 1.—G. B. | Achlorhydria and no pepsinogen 1944, 1945. Rennin absent in 1944, but present in 1945. |
| Case 2.—J. G. | Achylia 1940, 1943, 1944. |
| Case 3.—J. M. | Achylia 1941, 1942, 1943, 1944. Achlorchydria in 1945, and no rennin. Pepsinogen present in 1945. |
| Case 4.—J. C. | Achlorhydria and no rennin 1944. Pepsinogen present 1944. |
| Case 5.—M. A. | Achylia 1945. |
| Case 6.—J. D. | Achylia 1945. |
| Case 7.—H. G. | Free acid present in 1937, 1940, 1944. Achlorhydria 1936, 1938, 1943, 1945. Rennin and pepsinogen present 1945. |
| Vase 8.—H. A. | Achylia 1944. Achylia on one occasion in 1945. Pepsinogen present on one occasion. |
| Case 9.—J. B. | Achylia 1944, 1945. |
| Case 10.—W. K. | Achlorhydria 1943, 1944, 1945. Pepsinogen present 1944, 1945. Rennin absent 1944, 1945. |
| Case 11.—J. S. | Achylia 1943, 1944, 1945. |
| Case 12.—L. B. | Free acid and enzymes present 1945. |
| Case 13.—C. R. | Achlorhydria 2 × 1945. No rennin. Pepsinogen present. |
| Case 14.—J. C. | Achylia 1945. |

response. Erythrogenesis occurred and rose to normal levels when treatment was continued, regardless of the route of administration or of the clinical classification of the anemia of the selected test subjects. Tables II and III illustrate the early responses.

In two cases of Addisonian pernicious anemia, Moore *et al.* (6)

were able to obtain a clinical and hematological response. The initial red blood count (1.2 million) of one patient with true pernicious anemia, who received an oral dose of 100 mg. of liver *L. casei* factor for ten days, rose rapidly to a little over 3.0 million and remained constant at that level for four weeks. Reticulocytosis on the seventh day was 40 per cent. The second patient, also with

TABLE II

EFFECT OF PARENTERAL ADMINISTRATION OF "FOLIC ACID" ON PERIPHERAL BLOOD IN MACROCYTIC ANEMIA

| Case No. | RBC (Million) | | | Hb. (Gm) | | | Reticulocytes (Per Cent) | | | Dosage of Folic Acid | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Initial | 14 Days | Final Day | Initial | 14 Days | Final Day | First Day of Rise | Day of Peak | Per Cent at Peak | Daily Dose (mg.) | No. of Days | Total (mg.) |
| 1-A | 2.54 | 2.21 | 2.18 | 8.7 | 9.9 | 8.9 | 4 | 7 | 9.0 | 20 I.V. | 1 | 20 |
| 1-B | 2.16 | 2.69 | 4.08 (40) | 8.8 | 9.3 | 11.7 (40) | 4 | 8 | 14.8 | 20 I.M. | 18 | 360 |
| 2 | 1.82 | 2.23* | 2.92 (22) | 7.7 | 10.1 | 9.8 (22) | 4 | 6 | 10.4 | 50 I.V. | 1 | 50 |
| 3 | 1.95 | 2.59 | 3.22 (55) | 7.0 | 9.8 | 11.0 (55) | 6 | 8 | 6.4 | 20 I.V.<br>100(0) | 13<br>10 | 260<br>1000 |
| 4 | 1.96 | 2.92 | 3.54 (49) | 7.8 | 11.7 | 12.1 (49) | 6 | 8 | 14.6 | 20 I.V. | 26 | 520 |
| 5 | 1.61 | 1.58 | 1.58† (18) | 6.2 | 8.0 | 8.0 (18) | 7 | 10 | 11.0 | 20 I.V. | 26 | 520 |

* Folic acid was given after a suboptimal response to an experimental liver fraction, the last dose of which was given 11 days previously.

† Reticulogen, 50 U.S.P. units given from the eighteenth through the twenty-third day produced further reticulocytosis and maximal regeneration.

Addisonian pernicious anemia, was transfused with whole blood; the red blood count of 0.7 to 0.9 million rose to 1.4 to 1.5 million. This was followed by the daily oral administration of 30 mg. of liver *L. casei* factor for fourteen days. The reticulocytes began to rise on the fourth day of therapy; a maximum of 44.5 per cent occurred on the eighth day. The red blood count was 2.5 million on the fourteenth day. The original leukopenia and thrombocytopenia were corrected. The normal control subject who ingested 100 mg. of the same substance showed no change in reticulocytes, red cells, hemoglobin, leukocytes, or platelets during the three weeks of

TABLE III

EFFECT OF ORAL ADMINISTRATION OF "FOLIC ACID" ON PERIPHERAL BLOOD IN MACROCYTIC ANEMIA

| Case No. | RBC (Million) | | | Hb. (Gm) | | | Reticulocytes (Per Cent) | | | Dosage of Folic Acid | | | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Initial | 14 Days | Final Day** | Initial | 14 Days | Final Day | First Day of Rise | Day of Peak | Per Cent of Peak | Daily Dose (mg.) | No. of Days | Total (mg.) | |
| 6 | 2.01 | 2.69 | 4.22 (40) | 8.3 | 10.7 | 13.9 (40) | 4 | 6 | 14.8 | 100 | 23 | 2300 | Continued medication 100 mg. 2×week |
| 7 | 2.98 | 3.14 | 3.20 (20) | 9.3 | 8.3 | 9.2 (20) | 4 | 4 | 4.6 | 100<br>100* | 10<br>10 | 1000<br>1000 | |
| 8 | 2.59 | 3.14 | 3.72 (21) | 8.6 | 10.6 | 10.1 (21) | 4 | 7 | 14.4 | 100 | 22 | 2200 | Continued medication 100 mg. 2×week |
| 9 | 1.64 | 3.24 | 4.07 (19) | 7.1 | 10.2 | 9.8 (19) | 3 | 5 | 19.2 | 100 | 19 | 1900 | |
| 10 | 2.35 | 3.35 | 4.29 (48) | 7.8 | 9.9 | 12.4 (48) | 5 | 7 | 21.4 | 150 | 30 | 4500 | Continued medication 100 mg. 2×week |
| 11 | 2.32 | 3.31 | 3.38 (21) | 8.0 | 11.2 | 11.7 (21) | 4 | 7 | 19.6 | 100 | 22 | 2200 | Continued medication 100 mg. 2×week for 2 weeks, then daily |
| 12 | 2.01 | 3.57 | 3.82 (30) | 9.9 | 10.1 | 12.1 (30) | 6 | 8 | 10.0 | 100 | 21 | 2100 | Continued medication 50 mg. 2×week |
| 13 | 1.79 | 2.81 | 3.38 (30) | 8.7 | 11.3 | 13.0 (30) | 3 | 6 | 10.0 | 100 | 30 | 3000 | Continued daily medication |
| 14† | 2.97 | 3.23 | 3.55 (27) | 8.0 | 10.5 | 13.4 (27) | 3 | 3 | 6.6 | 100 | 27 | 2700 | Continued daily medication |

* Incubated with normal gastric juice before administration.
† This patient also received vitamin C, thiamine, and niacin.
** Final day indicated in parentheses.

observation. To a case of nontropical sprue they gave 20 mg. of liver *L. casei* factor each day for ten days and then 40 mg. every day for two weeks, intravenously. The red count of 2.6 million rose to 3.5 million and leveled off after twelve to fourteen days. The reticulocyte peak of 30.2 per cent was attained on the seventh day. Leukocytes and platelets returned to normal. Four months previous to the appearance of the nontropical sprue, the patient had had a hypochromic microcytic anemia. A case of pernicious anemia of pregnancy received 20 mg. of liver *L. casei* factor intravenously daily for ten days. There were 48.2 per cent reticulocytes on the seventh day and an increase of erythrocytes from 1.2 million to 3.0 million in fifteen days.

Late in 1945 Spies *et al.* (7) reported from Cuba the first results of oral administration of liver *L. casei* factor to nine selected cases of tropical sprue. The erythrocyte counts ranged from 1.5 million to 2.2 million. The red blood cells showed marked anisocytosis, poikilocytosis, and polychromasia. The hemoglobin values ranged from 5.6 gm. to 10.1 gm., and every reticulocyte count was less than 2.6 per cent. After treatment, the red cell counts ranged from 2.4 to 3.9 million, the hemoglobin from 10.7 to 13.8 gm., and the reticulocytes from 17.2 to 31.8 per cent. The reticulocyte peak occurred on the sixth to eighth day.

Darby & Jones (8) obtained appreciable reticulocyte responses in two patients with nontropical sprue by giving 15 mg. of liver *L. casei* factor intramuscularly daily. Later, Darby *et al.* (9) treated three patients with nontropical sprue with 15 mg. of liver *L. casei* factor daily, using the same route of administration. Again a satisfactory hematological remission was observed with an alleviation of the symptoms of nontropical sprue.

Doan *et al.* (10), in studying a virgin case of Addisonian pernicious anemia, showed that 2 mg. of synthetic *L. casei* factor injected daily produced a maturation of the marrow elements. They found that the reticulocyte crisis, the rapidity of increase in erythrocytes, hemoglobin, white cells, and thrombocytes, was similar to that following optimum liver extract therapy. These authors also substituted synthetic *L. casei* factor for liver extract in some patients who had developed sensitivity to liver extract and found it to be a safe and satisfactory substitute.

Zuelzer & Ogden (11) have shown that certain macrocytic anemias of infancy respond specifically to folic acid. They state

that there is a complete parallelism in the response to folic acid between the anemias of infancy and those of adult life.

Spies (12, 13) extended his studies to appraise further the value of synthetic liver *L. casei* factor in a variety of types of macrocytic anemia. Five cases of nutritional macrocytic anemia, five of Addisonian pernicious anemia, eight of sprue, three of macrocytic anemia of pregnancy, one of macrocytic anemia associated with chronic alcoholism, cirrhosis of the liver, and neuritis, and three of indeterminate etiology responded well to therapy with synthetic *L. casei* factor. The response in a typical case is illustrated in Fig. 3.

EFFECT OF FOLIC ACID ON MACROCYTIC ANEMIA OF SPRUE

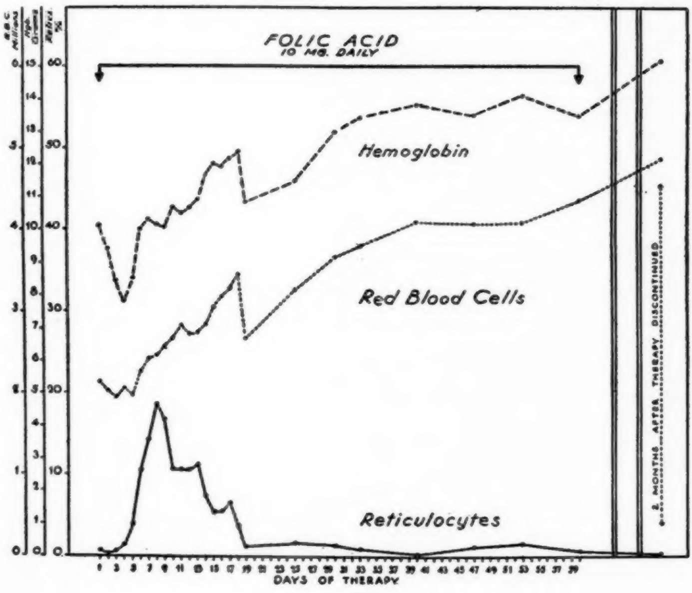


FIG. 3

Three cases of aplastic anemia, three cases of leukemia, and four cases of iron deficiency failed to respond. In five instances, liver *L. casei* factor had no effect until an adjustment in dosage was made; 10 mg. were substituted for the daily 5 mg. Two addi-

tional patients with macrocytic anemia, who had developed severe sensitivity to various forms of liver extract so that they were unable to tolerate amounts sufficient to maintain normal blood levels, were treated with liver *L. casei* factor. The hematological results were satisfactory, and no allergic reactions to therapy appeared. Spies found a number of patients who did not respond at a level of 3 to 4 mg. of the synthetic liver *L. casei* factor given each day orally. The same patients did respond to special liver extracts and to special yeast concentrates administered in daily amounts which contained 1 $\mu$g. or thereabouts of the liver *L. casei* factor. Spies is of the opinion that the antianemic factor present in liver extract is distinct from folic acid and that it and the special antianemic factor present in yeast, when they are isolated and tested, will be more potent chemical substances per unit of weight than folic acid. Even though all the diets he used were restricted for testing purposes, he stresses the opinion that a diet high in protein, minerals, and vitamins should be used in the treatment of macrocytic anemia in relapse.

Spies *et al.* (14), in substantiating the earlier observations of the response of sprue to liver *L. casei* factor, stress the remarkable antianemic effect of this substance. Though the authors maintain that they may not be justified in differentiating between nutritional macrocytic anemia and sprue until the etiology of both is better understood, they do make a diagnosis of sprue in the presence of acid steatorrhea, a symptom characteristic of sprue.

Clinical evidence for the hematopoietic value of synthetic *L. casei* factor is rapidly accumulating. Various investigators (15 to 24) have reported remissions in cases of macrocytic anemia.

The use of sternal puncture enables the physician to follow step by step the action of folic acid in the bone marrow. Profound transformations of the bone marrow elements occur within twenty-four hours. Whole islands of regeneration form, and gradually the megaloblasts decrease and the normoblasts increase. Cases associated with cirrhosis, aplastic anemia, and leukemia have not responded satisfactorily. While nutritional leukopenia responds well, Spies and his associates have learned that the leukopenia following amidopyrine or the sulfa drugs does not respond to folic acid. Likewise the postinfectious states and the associated leukopenia are not corrected by the administration of synthetic *L. casei* factor. Hence, it is obvious that precise diagnosis is the very basis for successful therapy in this field.

Milanes *et al.* (25) have made a thorough bacteriological study of the stools of twenty-five patients with tropical sprue and intestinal parasitism. The following criteria were used in the selection of these patients:

1. The patient must have glossitis.
2. He must have diarrhea, characterized by voluminous, foul-smelling, frothy, liquid, yellow stools; the stools must have also an increased fat content as determined by chemical analysis.
3. A body weight loss of at least twenty pounds must have occurred during the six months preceding the initiation of this study.
4. He must have a macrocytic hyperchromic anemia with a red blood count of 2.5 million or less and a color index of 1.0 or more; there must be megaloblastic arrest of sternal bone marrow.
5. There must be free hydrochloric acid present in the gastric juices on fractional analysis after histamine stimulation.
6. The oral glucose tolerance curve must be flat.
7. The blood calcium level must not be below 5 mg. per cent.
8. The serum amylase and lipase activity must be normal.
9. The patient must not have had specific therapy within the five weeks preceding this study.

Each patient was hospitalized and restricted to a diet containing no meat, meat products, fish, fowl, or cheese. He was allowed milk occasionally and one egg per day. Prior to treatment, base line clinical and laboratory studies were made by methods previously reported by Spies.

The feces from every patient were repeatedly examined. These investigators showed conclusively that the patients responded clinically and hematologically following the administration of folic acid despite the fact that no antiparasitic therapy was administered.

Hernandez Beguerie & Spies (26) made a series of roentgenlogic studies before, during, and after treatment with folic acid. Their criteria were similar to those of the preceding investigators. Each patient was hospitalized and received a daily diet which contained no meat, meat products, fish, or fowl. Repeated gastrointestinal x-ray series were performed on persons with sprue and on normal persons. The x-ray procedure was the same in each patient. The normal controls, as might have been expected, had entirely normal x-ray findings. The barium passed through the small intestine in a continuous connected column. This column was never fragmented or interrupted. Some of the patients with tropical sprue were used as positive controls and received no specific therapy during the course of the study. In these the barium

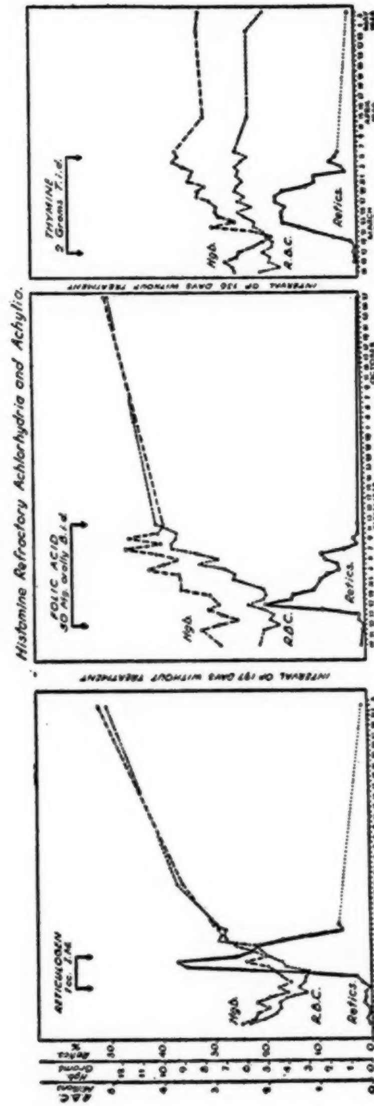
Histamine Refractory Achlorhydria and Achylia.
RETICULOGEN 1cc. I.M.
FOLIC ACID 50 Mg. orally B.i.d.
THYMINE 2 Grams T.i.d.
INTERVAL OF 197 DAYS WITHOUT TREATMENT
INTERVAL OF 130 DAYS WITHOUT TREATMENT
Hgb.
R.B.C.
Retics.
R.B.C. Millions
Hgb. Grams
Retics. %

FIG. 4

column was not continuous, due to many abnormal dilatations and spasms. These changes from the normal did not correct themselves and hence every barium series showed characteristic changes.

The stools were better formed, brown in color, and less copious and less liquid than those passed prior to treatment. The abnormal x-ray patterns observed in the patients changed dramatically after the administration of synthetic *L. casei* factor.

Unpublished observations by Garcia Lopez *et al.* (27) deal with the rehabilitation of the first eighteen cases of tropical sprue they studied. All of these cases have been discharged with essentially normal blood values. Fifteen of them went back to work. Three, who were over seventy years of age, had retired prior to their illness and following their illness had been a burden to their families. They are now able to care for themselves. Of the patients of Spies and his associates all who were under sixty years of age, who had uncomplicated nutritional macrocytic anemia or pernicious anemia and who had been treated adequately, also have been rehabilitated. A number who were over sixty years of age are not working but are no longer dependent on their families for their personal care. In the large series of cases of these investigators, they have noted a considerable number of patients whose red blood cells could not be brought up to a full five million. In every case where there has been a failure to bring blood values up to this desired level, liver extract subsequently has been given in large doses. In no instance has it caused additional blood regeneration. These patients were then given all the known synthetic vitamins but no blood regeneration was observed. Eight patients with macrocytic anemia had a definite iron deficiency, and it was necessary to give them iron before their blood values reached a normal level. They found that in some cases liver extract caused the red blood cells to increase a little more rapidly than folic acid in the dosage used, but apparently it did not raise them to a higher level.

Spies, as a result of many years of study, is convinced that persons with deficiency diseases have a disturbance of fundamental biochemical systems. Until more specific data can be accumulated, it is perhaps wise to omit unfounded speculation. Certain facts, however, should be stressed. There are certain other chemical substances that produce blood regeneration. Thymine (2,4-dihydroxy-5-methyl pyrimidine) is an integral part of nucleic acid, and Spies and associates have shown that it too has definite

antianemic effects on selected persons with macrocytic anemia. Frommeyer & Spies (28) have recently demonstrated that while its effect parallels those of a potent liver extract or synthetic folic acid, the response is at a much lower level (Fig. 4).

Spies *et al.* (29) have recently shown:

1. Two patients with Addisonian pernicious anemia in relapse did not respond to the oral administration of 10 gm. of synthetic uracil. These patients responded satisfactorily when they were given 10 mg. orally of synthetic *L. casei* factor. The synthetic uracil and the synthetic *L. casei* factor were each suspended in water and given every day for ten days.

2. Two patients with Addisonian pernicious anemia in relapse were given guanine, adenine, and pyridoxine (5 gm. of each) orally each day for ten days without response. Subsequently each of these patients responded well to the oral administration of 10 mg. of folic acid each day for ten days.

3. Two patients with Addisonian pernicious anemia in relapse and one patient with nutritional macrocytic anemia in relapse were given glutamic acid and *p*-aminobenzoic acid orally each day for ten days. Despite the fact that 5 gm. of each substance were given, there was no hematopoietic response. Thereupon, 500 mg. of xanthopterin were given intravenously to one of the patients each day for ten days, but again no response occurred. Both patients later responded spectacularly to the oral administration of 10 mg. of folic acid each day. These observations suggest that the body cannot efficiently, if at all, unite these components to form synthetic *L. casei* factor and thus induce a remission.

4. In a selected case of Addisonian pernicious anemia in relapse, 5 mg. of pteroic acid were injected intramuscularly per day for ten days. No response occurred. After injecting 5 mg. of synthetic *L. casei* factor intramuscularly each day for six days, striking blood regeneration was observed.

5. The daily oral administration of 100 mg. of alpha pyracin to a patient with Addisonian pernicious anemia produced no results. Lkewise, the same dosage of beta pyracin administered similarly to another patient was ineffective. Both patients responded maximally following the oral administration of 10 mg. of synthetic *L. casei* factor.

In November, 1945, Vilter *et al.* (30) initiated a study to test the efficacy of folic acid in maintaining persons with Addisonian pernicious anemia. Each of the twenty-four patients selected for

this study had been maintained under their observation for a number of years on liver extract. Nine months later the study was still in progress but the investigators do not believe it is possible to state with certainty whether or not the oral administration of folic acid can replace parenteral liver extract in the maintenance of such patients. Synthetic *L. casei* factor certainly does not always prevent the development of neural disturbances that occur frequently in persons with pernicious anemia. Studies such as this must be conducted for a number of years in order to provide us with the important information needed on this point.

Since December, 1945, Suarez *et al.* (31) have been attempting to determine whether synthetic *L. casei* factor administered orally can be substituted for parenteral liver extract in the treatment of old chronic cases of sprue. Despite gratifying results up to the present time, without further information these investigators believe that no final statement in regard to the use of synthetic *L. casei* factor in the maintenance of persons with sprue can be made.

As this manuscript goes to press, Spies reports that another *Lactobacillus casei* factor has been found by him to induce blood regeneration in a case of Addisonian pernicious anemia. This compound was obtained from a fermentation residue. Spies (32) concludes that natural fermentation *L. casei* factor produces no ill effects and does induce blood regeneration in selected cases.

It would seem well to consider that synthetic folic acid functions as a part of an enzyme system. It is well to keep in mind that the active principle in liver extract is not synthetic *L. casei* factor or any of the chemical substances mentioned in this review. When this substance is obtained in pure form, it will probably be much more powerful than any of the substances now available. The finding of a specific molecule such as *L. casei* factor that has such a profound effect on the elements of the bone marrow and probably on other tissue cells opens a fresh and fertile field for the clinical investigator who must now redefine Addisonian pernicious anemia, nutritional macrocytic anemia, pernicious anemia of pregnancy, macrocytic anemia of pellagra, and sprue in the light of all the various loose threads which enter into the meshwork of their pathogenesis.

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# NUTRITION

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For this review publications during the two-year period between October 1944, and October, 1946, have been considered. The volume of literature has been so great that it is impossible to review all of it adequately in the available space. Consequently, the discussion has been limited to human nutrition and to papers on animal studies which seem to contribute to human aspects. Publications for review have been restricted to those which have offered a definite contribution to knowledge, or which have indicated changes in trends of thought, or which have, unwittingly, retarded advancement in knowledge. A group of topics has been selected for convenience and the reasons for the choice will be apparent. It has seemed advisable to include a section on reports of nutritional observations made during and after the recent war, particularly those on persons subjected to severe deprivation. Some of these reports have provided unexpected information which will certainly change viewpoints of human nutrition.

## Nutrient Requirements

*Methods of study.*—There have been few significant contributions during the past two years in the determination of nutrient requirements. War experiences have cast doubt on results of laboratory procedures and a tendency to be guided by human experience has increased. It has been said frequently that inhabitants of Britain received supplies of ascorbic acid of about 25 mg. a day, that there was little evidence of deficiency, and that this amount satisfied requirements. Actually, this approach has been used for years. As the result of surveys, it has been revived in discussions of calorie requirements. Youmans *et al.* (1) listed average calorie intakes of subjects in Tennessee. Virtue & McHenry (2) made a similar compilation of a group of Canadian figures and concluded that some current statements of calorie requirements were too high. This approach to requirements is open to criticism because it often assumes that the subjects were in a state of health, an assurance which cannot be guaranteed. Mitchell (3) has ex-

plained clearly that the setting of precise figures should not be attempted and that requirements are not fixed amounts but ranges.

In recent studies of protein requirements several procedures have yielded useful data. Murlin *et al.* (4) developed a method for the study of protein availability which also permits an investigation of protein requirements. This involves "nitrogen-free" periods after intakes of various proteins at low levels. Endogenous nitrogen metabolism is determined by estimates of urinary nitrogen. Hegsted *et al.* (5) determined nitrogen requirements by ascertaining nitrogen balance on various levels of intake. They found that stabilization at a new intake was not obtained in three days and that earlier work may have given high results because stabilization was assumed to occur within that time. Darling and associates (6) determined protein requirements on the basis of physical well-being and ability to do work rather than on the more commonly used procedure of nitrogen balance.

The customary plan for determining the calcium requirement of children was described in two papers from the same laboratory by Watson and associates (7) and by McLean *et al.* (8). Using a few subjects the authors determined calcium retention with the assumption that calcium is not excreted into the intestine and that fecal calcium is the amount unabsorbed from the diet. Retentions by eight boys varied from 685 to 1165 mg. a day. This type of study has been ably criticized by Steggerda & Mitchell (9) who stressed the need for statistical analysis, particularly because of individual variations. Mitchell *et al.* (10) estimated calcium requirements by a procedure entirely different from the customary balance experiment. The authors compared the calcium content of one cadaver with the amount obtained by totalling calcium retention, as deduced from balance studies, and found the latter figure much greater than the former. They then concluded that customary estimates of requirement are much too great but they made no allowance for any possible period of negative balance. Several studies have emphasized the need for considering various dietary factors affecting calcium absorption and retention, factors which may be forgotten in constructing diets for use in determining requirements. The recent reports of the effect of phytic acid by Hoff-Jørgensen and associates (11, 12) may be cited as one example.

There have been no noteworthy contributions to the methods

for estimating requirements of iron, iodine, or of fat-soluble vitamins during the past two years.

Several types of procedure for studying requirements of water-soluble vitamins have been in use. The step-wise increase or decrease of intake to determine the amount necessary to give a particular urinary excretion, with or without a test load, or to give a certain blood level, has continued in use. Generally, elementary physiology has been overlooked: little thought has been given, for example, to renal threshold. The reviewers have seen no evidence that the sought-for urinary or blood levels were criteria of health. Several recent papers have contained observations which cast doubt upon this widely accepted type of procedure. Reinhold *et al.* (13) found that urinary excretion of thiamine could be altered by changing the carbohydrate-fat ratio in the diet. Mickelsen *et al.* (14) reported that it was not possible to distinguish between men on 1 or on 2 mg. intakes of thiamine by measuring urinary excretion. There was a two-to threefold variation for men within each group and the thiamine excretion was a characteristic of the individual and not of the intake. This observation could not be explained on the basis of fecal excretion, physical activity, basal metabolic rate, or body weight, but could have been due to renal threshold or kidney function. Greenberg & Rinehart (15) stated that the total thiamine in human blood is relatively static and is not affected rapidly by changes in intake. It should be pointed out that there are considerable data supporting the general procedure. Denko *et al.* (16) found that urinary output of thiamine promptly reflected a decrease in intake. Engelfried & McWilliams (17) reported data supporting the use of a test load in studying ascorbic acid metabolism. Kyhos *et al.* (18) stated that the renal threshold for ascorbic acid varied from 1.1 to 1.9 mg. per cent.

The effects of various levels of intake on health or on physical endurance have been used by several groups of workers to estimate nutrient requirements. This procedure has been used by Keys and associates (19) in studies of thiamine requirements, and by Johnson *et al.* (20) in determining ascorbic acid requirements. When such studies have indicated that N.R.C. Recommended Allowances are too great, the experiments have been criticized by some as being too short in duration. The correct length of study is a matter of opinion.

*Suggested requirements.*—The Recommended Allowances of the

Food and Nutrition Board of the U. S. National Research Council were fully criticized by Stare *et al.* (21). In 1945 (22) some revisions were made in the allowances, minor in the case of Calories, but more important in the downward revision of recommendations for thiamine and riboflavin. The allowances were defended and stipulations regarding their use were made by Wilder (23).

In the discussion of requirements, one of the most important contributions in the past two years, in the opinion of the reviewers, was that made by Mitchell (3) in his consideration of nutritional adaptation. With ample supporting evidence, Mitchell clearly indicated the undesirability of trying to set precise requirements. Requirements are not precise but are ranges. This was taken into account in a statement on requirements issued by the Canadian Council on Nutrition in 1945 (24). In a discussion of calcium requirements, Steggerda & Mitchell (9) pointed out the lack of need for a "margin of safety." They also showed that to feed all adults an amount of calcium to fit the needs of all individuals would require an excess above the average of 2.33 times the standard deviation. To supply this calculated amount would be uneconomical, if not impracticable. Strenuous efforts have been made to prevent the use of recommended allowances as "yardsticks of adequacy," but that use is likely to continue as long as allowances are stated in apparently precise quantities.

The question of whether requirements are stated too generously may seem of little consequence when the figures are used as goals for dietary improvement, but the matter is more than academic if such estimates are to be used in planning food supplies for large groups of people. It would be interesting to calculate the probable extravagance of supplying unneeded amounts of ascorbic acid or of riboflavin for a large army.

The few publications on calorie requirements during the past two years have dealt with evidence obtained in dietary surveys. The validity of such data is open to question. Youmans *et al.* (1) found that persons in Tennessee were able to maintain themselves on intakes much below recommended allowances. Virtue & McHenry (2) reported intakes for healthy children below allowances and concluded that calorie recommendations for adolescents were too great. A debatable point with regard to calorie requirements is whether activity classification is correct. A factory worker may be classed as moderately active but, because he sits most of the day

at an automatic machine, he may be more sedentary than an office person. Is a housewife always to be classed as moderately active?

These considerations make difficult the application of recommended allowances. Excess calorie consumption usually receives little attention in a discussion of requirements. Its importance as a nutritional abnormality leading to clinical problems has been described by Danowski & Winkler (25). They pointed out that a successful reduction in obesity can be accomplished only by re-education in basic eating habits.

In a study of resistance to exposure to cold, Mitchell and associates (26, 27) found that a high fat intake, particularly when taken in frequent small meals, was beneficial. The fat content of this diet constituted 74 per cent of the total calories, much greater than current conceptions of suitable fat intakes. An important series of studies on the relation of fat to the economy of food utilization has been reported by Forbes *et al.* (28, 29). In young rats, gains in body weight, in digestibility of protein, and in the retention of nitrogen and energy, were in the order of increasing dietary fat (for diets containing 2 to 30 per cent fat). In adult animals the dietary fat had no effect on the metabolizable energy of the diets, but did improve the digestibility and retention of food nitrogen. These studies give additional emphasis to the question of the proper amount of fat in the diet. The undesirable effects of a very high fat intake have been reported by Consolazio & Forbes (30). A group of men maintained on a diet in which 71 per cent of the calories was furnished by fat developed marked ketonuria, a loss in body water, salt depletion, an alteration in glucose tolerance, increased sensitivity to insulin, and a decrease in liver function.

Several studies on protein requirements have emphasized the generosity of popular recommendations. Darling *et al.* (6) maintained eight young men for two months on a diet supplying 50 to 55 gm. protein daily, mostly from vegetable sources, without alteration in the health of the subjects. In a careful summary the authors concluded that, under emergency conditions, a diet supplying 50 gm. protein, mostly from potatoes and grains, is satisfactory, at least for physically active young men. With nine young women as subjects Bricker and associates (31) measured protein requirements with proteins of different biological value. Requirements ranged from 43 gm. for milk protein to 74 gm. for protein from white flour. Hegsted *et al.* (5) determined protein requirements in

twenty-six apparently healthy adults; the report is particularly useful because of the statistical treatment of the data. The authors concluded that adults could obtain all of their protein requirements from cereals and vegetables. On such foods the minimum protein requirement. for a 70 kg. man was found to be 30 to 40 gm. daily varying with height of the individual. The requirement could be reduced 15 per cent by replacing one-third of the plant protein with animal protein. The authors stated that a suitable recommendation for protein would be 50 gm. a day. It should be noted that the results of these three studies are in good agreement and that the common conclusion should be considered in planning food supplies, especially in regions where protein sources are limited. Advocates of high-protein intakes have referred to the experiences of the Eskimos. Keeton *et al.* (26) raised the question as to whether the Eskimo used a high-protein diet to combat exposure to cold or because carbohydrate is not available. In tests on sixteen young men these authors found that a high-carbohydrate diet definitely increased tolerance to cold. One of their observations is of interest: subsistence on a high-protein diet required an average calorie intake of 162 per cent of basal energy to maintain weight; for the same purpose the intake of a high-carbohydrate diet was 191 per cent of basal. Talbot (32) has estimated the minimum protein requirement of children to be 0.6 to 0.7 gm. per kg. body weight per day, an amount much below the recommended allowance of 2.0 gm. per kg. Riegel *et al.* (33) studied the protein requirements for the maintenance of nitrogen equilibrium in patients following major surgical operations. Murlin *et al.* (34) studied the utilization of mixtures of amino acids. The biological value of a mixture of amino acids was found to be the same as that of intact protein, provided that allowance was made for the nonutilization of unnatural isomers. Albanese and associates (35 to 39) reported that *d*-arginine, acetyl-*d*-tryptophane, and *d*-cystine can be at least partially utilized by man, while *d*-histidine and *d*-tryptophane cannot be. Albanese (36) suggested that human utilization of *d*-amino acids is not an all-or-none phenomenon but is a matter of degree, decided by the competition of two processes: (a) the rate at which the organism can convert the *d*-to the *l*-isomer, and (b) the speed of urinary excretion. The investigations of Woolley (40) on strepogenin are of interest, particularly in studies of amino acid utilization and requirement. The absence of this peptide-like substance

might need consideration in studies using mixtures of pure amino acids.

Reference was made above to the attempt of Mitchell and associates (10) to calculate calcium requirements by comparing the total increment obtained by summation of retentions with the actual calcium content of a cadaver. McLean *et al.* (8) reported an average calcium requirement for seven preschool boys to be 300 mg. from nonmilk foods plus the amount contained in 478 gm. milk. The wide variation in individual requirements (685 to 1165 mg.) makes the validity of the average questionable. Steggerda & Mitchell (9) published a valuable contribution to the study of calcium requirements. The subjects were nineteen men. If two-thirds of the calcium was obtained from milk, or from an equally available source, the average utilization of dietary calcium was 32 per cent (coefficient of variation, 23 per cent). The average calcium requirement was 644 mg. $\pm$181 mg. daily. The effect of body size on calcium requirement was negligible within a moderate range. Steggerda & Mitchell concluded that a safe calcium allowance for adults who receive two-thirds of the total calcium from milk would be 10 mg. per kg. per day. This is reasonably close to conventional recommendations. Talbot (32) concluded that the daily calcium balance during the major part of childhood is about 175 mg., that children adapted to a low intake can absorb and retain 80 to 90 per cent of intake, and that the N.R.C. allowance of 1 gm. per day may be considerably in excess of minimum requirements for children.

Discussion of the effect of phytic acid on calcium absorption has continued. Hoff-Jørgensen and associates (11, 12) found that phytic acid decreased calcium absorption in young infants and in two 10-year-old boys. An unexpected finding was that two boys adapted themselves to phytic acid and that there was a decrease in its effect on calcium absorption.

In a two-year study of eight preschool children McKey *et al.* (41) found that phosphorus retention ranged from 6 to 16 per cent of the intake. About half of the amount excreted was in the urine. The authors concluded that intakes must have been ample or smaller excretions would have been observed. On the basis of calculated retentions, phosphorus requirements ranged from 3.5 to 10.8 mg. per kg. body weight.

Archdeacon & Murlin (42) investigated the effect of a low thia-

mine intake (0.27 mg. in a 3000 Calorie diet with 41 per cent of the calories from fat) on muscular efficiency and endurance. A decrease in endurance was evident in ten to fourteen days but there was no change in efficiency. In a study of much longer duration, Keys *et al.* (19) concluded that signs of thiamine deficiency are apt to appear within some months if the intake is less than 0.61 mg. daily, regardless of the calorie supply. The authors believed that an intake of 0.185 mg. per 1000 Calories was slightly less than adequate. This is in accordance with previous observations in the same laboratory (43) and is in good agreement with the conclusions of Holt (44) and of McHenry (45), based on summaries of evidence regarding thiamine requirements. Young (46) reported that a group of 385 men, women, and children showed no signs of clinical thiamine deficiency on intakes of 0.19 to 0.22 mg. per 1000 Calories. From observations on urinary excretions, with and without a load dose, and on blood levels in a group of young women, Oldham *et al.* (47) decided that a desirable intake would be 0.50 mg. per 1000 Calories. The data show that there was no real difference in blood levels of thiamine on intakes from 0.20 to 0.54 mg. per 1000 Calories. Large urinary excretions were interpreted as a sign of adequate intake; they could have been considered as evidence of excessive intake. In a study on tolerance to cold, Glickman and associates (48) found that an intake of 1.22 mg. thiamine in a diet of 3000 Calories appeared to be adequate. In a comparison of fecal and urinary excretion of thiamine by human subjects fed synthetic and natural diets, Hathaway & Strom (49) concluded that an intake of 0.84 mg. a day (0.4 mg. per 1000 Calories) from natural foods was adequate. Calculations of retentions of thiamine from their data show an average retention from natural foods of 0.33 mg. daily, or 0.15 mg. per 1000 Calories.

One of the most interesting recent papers on thiamine requirements is that by Roderuck *et al.* (50). These authors studied thiamine metabolism in lactating women and found that, even in the period of greatest thiamine secretion in the milk (two to three months' postpartum), the milk thiamine averaged 8 per cent of an intake of 1.1 mg. Since the urinary excretion averaged 23 per cent of intake, the authors concluded that the intake was adequate. The N.R.C. recommended allowance for lactating women is 2.0 mg. daily, an amount considerably in excess of the quantity considered to be adequate by Roderuck *et al.* A review of recent litera-

ture leads to the conclusion that even the revised thiamine allowances of 1945 are still too great.

A short study of riboflavin excretion in a few subjects by Hathaway & Lobt (51) illustrates some of the difficulties encountered in determining requirements. On a synthetic diet supplying 1.09 mg. a day, subjects excreted 0.47 mg. (0.16 in the urine and 0.31 in the feces). On a natural diet containing 1.33 mg. daily, subjects excreted 1.32 mg. (0.20 mg. in the urine and 1.12 in the feces). Presumably, there was intestinal synthesis of riboflavin or else the subjects absorbed only 16 per cent of the riboflavin in natural foods. Proceeding on the assumption advanced for thiamine by Hulse *et al.* (52), that the excretion level of a vitamin reflects the tissue concentration, Davis and associates (53) measured urinary excretions of riboflavin of thirteen young women on various levels of intake. The conclusion was that the riboflavin requirement of healthy young women is about 0.50 mg. per 1000 Calories. During the secretion of mature milk by lactating women, Roderuck *et al.* (54) found that the riboflavin in the milk ranged from 3 to 15 per cent of a dietary supply of 3.1 mg. a day. During the same period the urinary excretion was from 26 to 61 per cent of the intake, with an average of 43 per cent. The conclusion could be drawn that the intake, which was about the same as the recommended allowance, was considerably in excess of need. From a study of Royal Air Force personnel, Macrae *et al.* (55) found that with an average intake of 1.9 mg. riboflavin per day there were no signs of deficiency. They concluded that the average requirement for adults does not exceed 2 mg. per day.

Briggs *et al.* (56) studied the effects of nicotinic acid restriction in two subjects. An intake of 3 mg. daily was considered to be slightly less than the minimal requirement. An interesting paper by Frazier & Friedemann (57) attempts to correlate requirements for nicotinic acid with the dietary composition in respect of other nutrients. The basic data were calculations of protein, thiamine, riboflavin, and nicotinic acid contents of 1863 dietaries recorded by Goldberger and others. From these data the following conclusions were drawn: (*a*) the minimum daily intake of nicotinic acid needed in a marginal diet containing corn products is 7.5 mg.; (*b*) if the diet contains a high level of good quality protein, riboflavin, or green vegetables, the nicotinic acid requirement is reduced to 5 mg.; and (*c*) on a corn-containing diet supplemented

by large amounts of milk, or on a diet devoid of corn, the minimum nicotinic acid requirement is about 4 mg. daily. The effect of a liberal intake of corn in increasing nicotinic acid requirements has been reported by Krehl *et al.* (58). This action of corn was counterbalanced by adding tryptophane to the diet (59). Woolley (60) stated that corn contains a pellagra-producing constituent which appears to be a weakly basic, water-soluble compound. It would appear that there is justification for calling pellagra a corn-eater's disease.

The determination of B-vitamin requirements is complicated by possible synthesis in the intestine. Najjar *et al.* (61) reported the apparent synthesis of riboflavin in human subjects and the consequent prevention of deficiency symptoms. In an editorial the *Lancet* (62) called attention to the anticipated effect of sulfaquanidine therapy in causing deficiencies by prevention of synthesis. Observations by Ellinger & Benesch (63) supported the editorial. Schweigert *et al.* (64) found that diets containing lactose, sucrose, or dextrin were not conducive to thiamine synthesis in rats but riboflavin was synthesized on lactose diets. Obermeyer and associates (65) stated that fecal output of riboflavin was dependant upon the quantity of feces. The possible biosynthesis of nicotinic acid in man was described by Najjar *et al.* (66) but no data were provided to prove that synthesis occurred. Hardwick (67) reported the production of a "pellagrous" state in one patient by sulfaguanidine administration. It is debatable as to whether vitamins synthesized in the intestinal tract can be absorbed. Emerson & Obermeyer (68) found in rats that thiamine in the feces, at least under some conditions, was largely present as cocarboxylase and that it could not be absorbed. Some strains of viable yeast were reported by Ness *et al.* (69) to prevent the absorption of thiamine when the yeast was ingested by human subjects.

Kline & Eheart (70) reported ascorbic acid "saturation" studies on nine normal young women. A subject was said to be saturated if 50 per cent of a 400 mg. test dose of ascorbic acid was excreted in the urine. It was claimed that seven of nine subjects were saturated on an intake of 1.8 mg. ascorbic acid per kg. body weight per day. Actually, one subject, who was said not to be saturated on that intake, excreted 40 per cent of the test load and 34 per cent on an intake of 2.2 mg. per kg. per day. The subject was unsaturated on an intake of 200 mg. per day, on the basis of the criterion used. The results among the small number of subjects were so varied as

to be inconclusive. Shields *et al.* (71) found that the total twenty-four-hour excretion of ascorbic acid in the sweat of subjects kept in a hot, moist room was less than 3 mg., a negligible fraction of the intake. They found some correlation between urinary volume and output of ascorbic acid. Johnson *et al.* (20) concluded from a study of twenty-four young men that an intake of 75 mg. ascorbic acid daily maintained and, in some cases, increased body stores, as judged from measurements of plasma levels and the results of saturation tests. From an investigation of the relation of ascorbic acid to gingivitis, Linghorne and associates (72) found that intakes of 75 to 80 mg. daily were required to give a high level of the vitamin in the plasma or white cells and to prevent the recurrence of gingivitis in mouths which had been treated to eradicate the infection at the start of experimental feeding. In contrast to the above contributions which support a high requirement for ascorbic acid, the careful work of Pijoan & Lozner (73) with six subjects studied for six months demonstrates clearly that the minimum requirement for adults is 18 to 25 mg. per day. They acknowledged that the amount required for saturation, as represented by urinary excretion, is 80 to 100 mg. but they regarded this level as a dietary excess, definitely above needs. Their finding has been supported by Najjar *et al.* (74), who maintained seven young adults on a daily intake of 25 mg. for eighteen months without clinical evidence of deficiency. These observations on ascorbic acid requirements have been amply confirmed by experience in Great Britain and elsewhere during the war.

This discussion of nutrient requirements can be closed fittingly by a quotation from a report by McDaniel and associates (75) on observations on prisoners-of-war:

> "It seems justifiable to theorize that vitamin and other nutritional requirements have been placed too high, and that humans do adapt themselves to abnormally low levels of nutritional intake."

## Dietary Surveys

A summary of methods usually employed in dietary surveys has been published by the Nutrition Society of Great Britain (76). McHenry *et al.* (77) have discussed sources of error in dietary studies. Variations in food intake from day to day and from week to week in persons with free choice is an important cause of error, especially in short period surveys.

The proportion of various nutrients contributed by a midday

meal to the total intake of thirty-nine boys was ascertained by Cook and associates (78). The school meal provided 36 to 42 per cent of total calories, protein, thiamine, calcium, and iron; 58 per cent of ascorbic acid and 69 per cent of vitamin A. Bray *et al.* (79) described the contribution to the total intake of school children made by school meals. Since a general recommendation is frequently made that a school meal should provide one-half of a day's requirements, it is worth noting that Bray and associates found that children could not take one-half of the calorie requirement at the noon meal. The effects of school lunches upon the general intake were also studied by Bransby & Wagner (80). These authors found that a poor school meal worsened the general intake while the converse was also true. The study was made on four-hundred children in the fall of 1943 and three-hundred in the spring of 1944 in two English industrial towns. Very little day-to-day variation was found. When compared with League of Nation standards, the supplies of nutrients were fairly adequate except in the case of calcium, which was low because of the small supply of milk.

In an interesting discussion of food intakes in Jamaica, McCulloch (81) calculated that three million acres would be required to raise food to provide adequate supplies for the inhabitants; the amount of arable land in the island is 700,000 acres. The recommendation to develop more mixed farming and stock-raising was probably based on prevalent ideas about need for animal protein, upon which recent investigations have cast doubt. Milam & Bell (82) determined the food sources of nutrients for a North Carolina village. Ten foods supplied 85 per cent of each nutrient. It should be noted that bread supplied 20 per cent of calories and was the chief source of nicotinic acid and of protein, while dried peas and beans were the principal sources of thiamine and iron.

Stevenson *et al.* (83) studied food intakes in Canadian military hospitals. The average Calorie value of food as served to the patients was 2700. Consumption ranged from 1800 to 2300 Calories and it was noted that one-half of the patients lost weight while in hospital. Kitchen wastage was from 1400 to 2300 Calories per person per day. Factors responsible for the unsatisfactory situation were: the attitudes of medical officers and of patients (caused by a lack of nutritional information), servings made small to reduce costs, and improper spacing of meals and supplements with deleterious effect on appetite.

Another detailed report gives information regarding food con-

sumption in 2300 households in Australia in 1944 (84). With the exception of calcium, supplies of various nutrients were fairly satisfactory. Supplies of vitamin A varied considerably from one region to another. Meat intake was much greater than in Britain, U.S.A., or in Canada, while potato consumption was much less.

Water, a source of nutrients commonly overlooked in dietary studies, was discussed by Murray & Wilson (85). Hard water may contribute as much as 200 mg. calcium a day. Goiter is not likely to be found in areas in which the iodine content of the drinking water is greater than 3 μg. per liter.

Evans & Lubschez (86) contrasted food habits of a group of children in New York in 1917 with those of a similar group in 1942. Generally speaking, an improvement was apparent, particularly with regard to milk, fruits, and vegetables, but the authors believe that protective foods were still not used adequately.

A very thorough study of food consumption by American soldiers quartered in the U.S.A. was reported by Howe & Berryman (87).

A number of other dietary surveys, made in conjunction with studies on nutritional status, will be discussed in a later section.

## Determination of Nutritional Status

*Method.*—This subject has been reviewed capably and critically by Dann & Darby (88). Study of that review should help to clarify the present unrealistic situation in human nutrition. General aspects of nutrition assay have been considered in several papers. For children, Kornfeld & Nobel (89) advocate the Pelidisi index of von Pirquet, a procedure not looked upon favorably in North America. The application of statistical methods to nutritional surveys is recommended by Wiehl (90), particularly with regard to the calculation of the magnitude of errors. However, underlying the advisability of statistical treatment is the question regarding the value of appraisal procedures. Yudkin (91) has discussed the general problem and has pointed out particularly the lack of specificity of tests now available. A committee of the American Medical Association (92) has published a table listing the stigmata of deficiencies; this table is indefinite and fails to emphasize the insecurity of many of the stigmata. Biochemical procedures have been discussed by several groups, particularly with regard to the determination of vitamins in blood and urine.

Johnson *et al.* (93) conclude that fasting specimens are prefera-

ble to loading tests, that blood specimens may be taken at any time of the day, and that random specimens of post-prandial urine should not be used. In a separate paper Johnson and associates (94) compared intravenous and oral vitamin-load tests. For routine survey work the authors recommend, in place of long interval sampling, the collection of urine for one hour after an intravenous dose and for two hours after an oral dose. The paper includes no discussion of the significance, if any, of the urinary levels. As an example of the value of urinary and blood levels, Harris *et al.* (95) reported amounts found in 350 "deficient" persons and in 70 controls, significant differences between the two groups being evident in the average plasma levels of vitamin A, carotene, ascorbic acid, and tocopherols. No doubt there was a difference in averages, but the averages were misleading because of the very wide ranges of values. In the case of ascorbic acid, higher individual values were found in the deficient than in the control group. The reported levels did not confirm the existence of deficiencies in the area of Birmingham, Alabama, but did demonstrate extraordinary individual variations. In contrast to these reports on vitamin tests in blood and urine, note should be made of the report of Berryman and associates (96). The principal conclusions from this study were: (*a*) there is a relation between the immediately preceding dietary intake of a given vitamin and the average fasting urinary level of the same nutrient in a group of subjects; (*b*) fasting urinary levels should not be used, alone, to diagnose nutritional conditions; (*c*) there is no statistical correlation between fasting urinary levels and load test response; and (*d*) there is no assurance that all subjects in a group will have the same range of excretion on the same intake levels.

From a brief study of six subjects, Hulse *et al.* (52) decided that measurements of thiamine excretion reflect changes in the concentration of freely diffusible, extracellular thiamine and that determinations of blood pyruvate might be useful to detect thiamine deficiency but that both estimations are applicable only to essentially normal persons in whom normal baselines of renal function and of pyruvate formation may be assumed. Gifft & Hauck (97) studied four procedures for detecting thiamine deficiency by determinations of urinary values. It was said that each subject of four in the group behaved consistently, but there was very evident lack of agreement in the results. A critical examination of the paper

gives the impression that all four procedures were valueless. A rapid method for the estimation of thiamine in urine was described by Perlzweig *et al.* (98) and recommended for use in nutrition surveys.

Several reports on the determination of hemoglobin have appeared in Great Britain. Special investigations were made on procedures. Macfarlane *et al.* (99) discussed the Haldane hemoglobinometer and stated that the standard value for that instrument was 14.8 gm. per cent. They found good agreement between results obtained by the Haldane and by the alkaline hematin procedure. The accuracy of the latter method was studied by Peterson & Strangeways (100). There is still need for agreement in the expression of hemoglobin values and for realization of the inaccuracy of some commonly used methods.

The phenylhydrazine procedure for ascorbic acid was found by Pijoan & Gerjovich (101) to be satisfactory for blood, possibly for urine, but unreliable for tissues in which various interfering substances occur. The lack of specificity of gum lesions as a criterion of ascorbic acid deficiency was stressed by Stamm *et al.* (102). The difficulties of studying ascorbic acid nutrition by excretion and load tests were well illustrated in the work of Meyer & Hathaway (103) on eight young children. With a basal diet supplying 25 mg. a day, the average excretion was 7 mg. and load tests were said to show unsaturation of tissues. On an intake of 31 mg., excretion averaged 9 mg. and the tissues were considered to be saturated. One might well ask why the subjects excreted 28 per cent of an intake which was too low for saturation.

## Deficiency Diseases and Nutritional Status

The relation of vitamin A to skin diseases was investigated thoroughly by Leitner & Moore (104). There is an apparent relation in the case of a few rare skin conditions but the authors conclude that skin lesions commonly seen are not related to a deficiency of vitamin A. Hamilton *et al.* (105) found no relation between vitamin A intake and color vision. In common with several reports in the past five years, Anderson & Milam (106) found no relation between the incidence or severity of conjunctival lesions with either blood levels or dietary supply of vitamin A. Conjunctival lesions were found to increase with age.

Since the occurrence of conjunctival thickening is still said to

be due to vitamin deficiency (92), note should be made of the careful study reported by Robertson & Morgan (107). Healthy young women, all showing conjunctival thickening, were divided into two groups. For one year one group received 50,000 I.U. of vitamin A per day while the other group was given corn oil. The supplements were without effect on the conjunctivae, and it was not possible to select members of groups by ocular examination. The authors found some relation between conjunctival thickening and exposure to dust and other irritants; they concluded that conjunctival changes have no relation to vitamin A intake and function. Sound evidence is now against the use of conjunctival thickening as an index of vitamin A deficiency.

The still controversial problem of corneal vascularity and other eye abnormalities in relation to ariboflavinosis has been discussed in a number of papers. In a study on rats Lee & Hart (108) found that riboflavin deficiency caused a decreased oxygen uptake in corneal epithelium, and that the oxygen uptake of the stroma was elevated when vascularization was present. Several reports (109 to 112) from various laboratories are in agreement that corneal vascularity may be caused in various ways (deficiencies of riboflavin, vitamin A, or of several amino acids, trauma, and irritation). This lack of specificity of corneal changes as a sign of riboflavin deficiency was reviewed by Anderson & Milam (113) who found no relation between vascularization and dietary riboflavin or other alleged signs of riboflavin deficiency. Fish (114) stated that ocular rosacea is not due to lack of riboflavin. Spies *et al.* (115) reported a good correlation between ocular disturbances, mostly inflammation, and riboflavin deficiency. McCreary and associates (116) summarized the present situation regarding this alleged sign of riboflavin deficiency with a statement which merits repetition:

> "A uniform peripheral corneal vascularization is not a safe basis for a diagnosis of riboflavin deficiency existing at the time of examination. Such a lesion may be due to riboflavin deficiency but the deficiency could have occurred at any time previous to the examination. Also these blood vessels could have been reactivated by some cause other than the lack of riboflavin."

Cayer *et al.* (117) stated that cheilosis and glossitis were the most reliable evidence of B complex deficiency, that cheilosis is not usually due to riboflavin deficiency but to lack of nicotinic acid, although cheilosis may be caused by trauma as from poorly fitting dentures, that glossitis is not a specific sign of nicotinic acid de-

ficiency, and that both signs are more likely to be due to a multiple deficiency. The lack of value of determinations of methyl nicotinamide in urine as an index of nicotinic acid deficiency has been reported by Mickelsen & Erickson (118), by Ellinger & Benesch (119), and by Coulson *et al.* (120). The last authors favor a load test with estimations of nicotinic acid.

A number of papers have dealt with hemoglobin levels in population groups. Yudkin's (121) study on young women in Britain indicates that about 14 gm. per cent may be a normal average for such persons. A comparison of hemoglobin levels in Aberdeen, Scotland (122) showed less anemia in preschool children, somewhat more in those of school age, more in adolescent girls, and about the same amount in working class adults in 1945 as compared with 1935. Quite different results were reported by Davidson *et al.* (123) who found less anemia among British children in 1944 than in 1942; the improvement was ascribed to the use of higher extraction flour. Dobbs and associates (124) found improvement in hemoglobin levels in British children from 1942 to 1943. In another study of British children Mackay *et al.* (125) found a relation between hemoglobin values and economic conditions. Hemoglobin levels for 2168 white persons and 861 negroes in North Carolina were examined statistically by Milam & Muench (126). In general, observed values were well described by normal frequency curves but a small number of women of both races were below the range of the distribution curve. Presumably, these women could have been described as anemic. Mean values for various groups arranged according to race, sex, and age ranged from 12 to 14 gm. per cent.

As part of a comprehensive survey in Tennessee, Youmans *et al.* (127) reported that dietary information, evaluated in terms of N.R.C. Recommended Allowances, indicated considerable lack of calcium in Tennessee diets. Of the children under three, 25 per cent showed clinical or laboratory evidence of rickets. There was little other evidence of vitamin D and/or calcium deficiency. Forty-eight per cent of the subjects reported upon in another paper (128) had intakes of vitamin A which were presumably deficient. Measurement of dark adaptation showed considerable deficiency with fair correlation with the dietary observations. In a number of persons blood vitamin A levels were low; there were few actual signs of deficiency and these did not correlate with intake,

blood values, or with adaptometer readings. For no clear reason the authors concluded that blood vitamin A determinations provide useful evidence of deficiency, a conclusion at variance with the finding of Aron and associates (129) that the blood levels of vitamin A and of carotene are an expression of liver function.

An interesting account of riboflavin supply and excretion in a group of male prisoners has been given by Hagedorn *et al.* (130). For two years the men received about 0.5 mg. riboflavin a day, of which they excreted 0.05 to 0.12 mg. Supplements of the vitamin were tried and caused an increased excretion but had no effect upon cheilosis, changes in the naso-labial skin, nor on lesions of the sclerae. These lesions were more common among the supplemented subjects than in those with low intakes.

In a study of African children admitted to hospital with pellagra, Gillman & Gillman (131) found fatty livers. Desiccated stomach given with hydrochloric acid caused prompt recovery. The authors report that cirrhosis is common in Africa and it may be due to repeated exposure to malnutrition.

From ascorbic acid saturation tests on forty pregnant women Craig *et al.* (132) found these subjects became saturated more slowly than nonpregnant persons on the same intake. A similar study on adults in northeast England in early 1943 (133) indicated that women were less well saturated than men.

Leeson *et al.* (134) reported that patients in a mental hospital accustomed to an intake of about 25 mg. daily were not improved in any respect, except in plasma levels, by a three-month supplementation with ascorbic acid. The supplements produced no reduction in gingivitis. The authors interpreted the result as indicating that 25 mg. was probably an adequate daily supply. In a study of 747 whites and 377 negroes in Tennessee, Youmans and associates (135) found that 20 per cent ofwhite persons and 33 per cent. of negroes had intakes of less than 30 mg. ascorbic acid a day. There was no scurvy and no cutaneous hemorrhages but there were unspecific signs of deficiency. A few persons had serum levels below 0.3 mg. per cent but there were no zero values. There seemed to be partial correlation between gingivitis and serum levels and between the latter and intakes.

Walsh (136) recorded observations on a large group of men in a prisoner-of-war camp in Germany. Fifty-two men who had trench mouth or gingivitis were contrasted with a group of "normals"

who were without the mouth lesions. There were no differences in urinary excretion before and after a test dose of ascorbic acid. The mean ascorbic acid intake during 1944 was 39 mg. and urinary levels were low but health in the camp was excellent and gingivitis disappeared without dietary change. A different viewpoint was presented by Linghorne and associates (72) who reported that a high intake of ascorbic acid prevented the recurrence of gingivitis in subjects who had been cleared of infection by local treatment. Of 366 subjects in Puerto Rico, Munsell *et al.* (137) reported that 58 per cent had plasma ascorbic acid values in the "severe deficiency range." None of the subjects had any of the symptoms usually associated with scurvy. The authors were highly critical of the use of plasma ascorbic acid values as an index of deficiency and the data amply justified their attitude.

A number of studies on school children have been reported. Examination of two thousand school children in Florida (138) showed a high incidence of the popular, indefinite signs of minor deficiencies (roughened skin, angular stomatitis, etc.). Two large studies in England were concerned with supplementation of ordinary diets with vitamins. In Cambridge (139) a slight effect of the supplements was seen and Yudkin postulated that failure to secure more improvement may have been due to suboptimal intake of other nutrients. The other study, conducted by the British Ministry of Health (140), showed no effects caused by the supplements. The obvious conclusion from both studies is that the vitamin intakes of the children were already optimal.

Milam and associates have described several surveys in North Carolina and these are noteworthy because of conservative interpretations. In Alamance County (141, 142) families were using food supplies low, on the basis of N.R.C. Recommended Allowances, in thiamine, ascorbic acid, and nicotinic acid, but the most prevalent nutritional abnormalities were obesity, underweight, and low hemoglobin values. No correlations were found between conjunctival changes and vitamin A intakes or plasma levels, between corneal vascularization and riboflavin supplies, or between gingivitis and plasma ascorbic acid. Observations in Wayne County (143) were very similar and the authors concluded that there is a broad zone of intakes below Recommended Allowances in which individuals can adjust themselves without patent signs of deficiency.

One of the most widely publicized nutrition surveys in recent years was that conducted on 868 persons in a small sector of Newfoundland by Adamson *et al.* (144). From data on food imports, supplies of nutrients were calculated and were said to be deficient in calcium, ascorbic acid, vitamin A, thiamine, and riboflavin. High general death rates, high infant mortality, and a considerable incidence of tuberculosis were ascribed to poor nutrition. Possible lack of prenatal care or of hospital facilities were overlooked in the desire to support the general contention. "In attitude and behavior the children resembled little adult men and women," but perhaps Newfoundland children behave differently than do children in large cities. Children were not subnormal in height. The authors reported that muscular development was very poor in many subjects of all ages and both sexes and that native Newfoundlanders were unable to do manual work in the building of airfields. The present reviewers have yet to see a nutritionist, however well nourished, who could stand the arduous work of fishing off Newfoundland; 40 per cent of Newfoundland families are dependent upon fishing for a livelihood. The authors noted that florid beriberi had almost disappeared from the island and there were no cases of pellagra. Signs of rickets were seen in 3 per cent of the children.[1] The argument for poor nutrition in Newfoundland was largely based on prevalence of unspecific minor signs and symptoms, such as stary hair, conjunctival thickening, cheilosis and hyperemia of the gums. One may well ask, what was the real state of health of the persons examined?

A Newfoundland survey of a somewhat different nature and with more conservative interpretations was conducted among thirty-nine families in one village by Metcoff and associates (146). The study was restricted to those families in which there had been a pregnancy in the preceding two years. A study of food habits showed that infants received an adult diet after weaning, and that there was a small consumption of milk, fruit, and vegetables, with the exception of potatoes. Children were reported to be undersize by U. S. standards and 60 per cent of the women were anemic. There was no pellagra, no objective findings of thiamine deficiency, and no evidence of ascorbic acid deficiency, unless gum changes were used as criteria. As in the other Newfoundland study, the

[1] They were observed in 9 per cent of a group of Ontario children reecntly examined (145).

chief signs of nutritional deficiencies were the unspecific and unproven ones of vague eye symptoms, conjunctival thickening, and gum abnormalities. The authors applied a conservative interpretation to this evidence.

After giving a grim account of food supplies available to Indians in northern Manitoba, Moore *et al.* (147) reported having seen no single classical deficiency disease. "However, every Indian observed had some abnormality of the conjunctivae, ocular limbic blood vessels, tongue or gums." Poor nutrition was blamed for high morbidity and mortality rates and for physical deterioration (physical measurements were not taken). This report contains a criterion of physical deterioration not hitherto employed in nutrition surveys, the sizes of shirts and pants. Apparently, Indians forty years ago wore pants sized 38 to 44 and now the waist lines have shrunk to size 34 to 38. Did it not occur to the authors that obesity might be less frequent?

In striking contrast to the Newfoundland study first discussed and that among Manitoba Indians was that conducted among Indians in Mexico by Anderson *et al.* (148). The district was described as one of the most depressed in Mexico with a high death rate, poor houses, and no sanitation. Practically all edible plants were used as foods and cooking was simple and not excessive. Calorie intakes were low and little fat was used. Only small amounts of animal protein were available. At least one-third of the persons were underweight and very few were overweight. Signs of vitamin A deficiency were few and indefinite and, once again, there was no correlation between conjunctival changes and intake or blood level. Riboflavin supplies were poor and there was considerable angular stomatitis and cheilosis. While intakes of nicotinic acid were small, there was no pellagra. Despite good supplies of ascorbic acid, gingivitis was common and there was no correlation between the occurrence of gingivitis and intakes or blood levels of ascorbic acid. Children were of small size and underdeveloped. The authors stated that there was surprisingly little definite clinical evidence of nutritional deficiencies. In another Mexican study Calvo and associates (149) reported observations on several hundred individuals from economically poor families in Mexico City. The families received all of their meals in a government-operated dining room for thirteen months. The meals were adequate but there were no changes in body weight, hemoglobin, blood protein, bleeding gums,

corneal vascularization, and dry skin. The only improvements observed were in respect of photophobia, painful tongue, gingivitis (swollen, reddened gums) and glossitis. The authors concluded that the disturbing failure to demonstrate more improvement was due either to a better initial status than was assumed, or to failure of existing procedures to detect mild deficiencies. Probably both factors were operative.

Platt (150) has given a comprehensive report on nutritional conditions in the British West Indies. While conventional signs of deficiency were used as criteria, Platt pointed out that some of them (e.g., xerosis conjunctivae, loss of elasticity of skin) are not specific. The principal dietary shortages were riboflavin, calcium and protein. Heights of children were good but weights were low. Dental defects were common. There was little evidence of lack of vitamin A, of rickets, or of gingivitis.

Two recent reports have dealt with nutritional conditions in Iceland. Dungal (151) found that 77 per cent of a group of children in Reykjavik showed some evidence of having had rickets. A more general report (152) described food habits. Protein intakes were high, but supplies of calcium, ascorbic acid, and vitamin D were low.

The possible relation of nutritional status to mental development in children has received some attention. In an indefinite paper, Kugelmass *et al.* (153) claimed to have found that an increase in I.Q. was associated with an improvement in nutritional status. A somewhat similar report was given by MacKenzie (154); the mental ability of the children was graded by the teachers as bright or slow. Harrell (155) has described a two-year investigation of the effects of added thiamine on a group of about 120 children. The conclusion was reached that the extra thiamine produced significant increases in learning ability and that "the cumulative effects throughout a lifetime may spell the difference between alert, successful living and a marginal ineffectiveness." A critical examination of the data fails to show that the findings support such a sweeping conclusion. Of the total number of tests applied, the extra thiamine was claimed to produce a significant effect on one-half, and some of the "significant" responses are peculiar. It would not have been anticipated that thiamine would improve visual acuity in the left eye and not in the right one. More convincing evidence than this would be required to justify the supply of extra thiamine to all school children.

In the opinion of the reviewers a discussion of recent nutrition surveys would be incomplete without reference to the address given by Sinclair (156) to the American Public Health Association in 1944. Sinclair pointed out that dietary surveys are expensive, inaccurate, and the results cannot be evaluated because of a lack of information regarding requirements. He stated that a plasma ascorbic acid value of zero has not been proven to indicate ill-health, and that he knew of no analysis of urine in the course of rapidly conducted surveys which could provide useful information about the state of nutrition. A sentence deserves quotation: "Fed by inadequate evidence a fire of uncritical enthusiasm to detect and treat imagined deficiencies has swept our countries, its flames fanned by certain political and vested interests, and when the scientists have left their laboratories, they have not always lent a hand at the fire-engine."

## Clinical Use of Nutrients

The most outstanding advance in the therapeutic use of nutrients in the past two years has been the recognition of the effect of folic acid in macrocytic anemias. The isolation and synthesis of folic acid made possible its clinical trial in anemic patients, a use suggested, no doubt, by investigations over a period of years on nutritional anemias in monkeys and in man. Spies and associates (157) published a preliminary report and, subsequently, Spies reported (158) a series of forty-two cases of macrocytic anemia treated with folic acid. All the patients had a red cell count of not more than two and one-half million and a color index over one, were previously untreated, and showed a megaloblastic bone marrow with typical erythroblastic arrest. They were given a restricted diet and folic acid in doses of 10 to 50 mg. daily, oral or parenterally. Twenty-six responded to the treatment: five cases of nutritional macrocytic anemia, five cases of Addisonian pernicious anemia, eight cases with sprue, three with macrocytic anemia of pregnancy and one with chronic alcoholism and hepatic cirrhosis, one with carcinoma of the stomach, and three of undetermined etiology. Treatment was ineffective in one case of nutritional macrocytic anemia, and in cases of aplastic anemia, leukemia and iron-deficiency anemia. In those who responded, there was immediate subjective improvement with prompt hematopoietic response within three to ten days. A dosage level of about 20 mg. daily was

thought to be effective. Goldsmith (159) has also reported on the use of folic acid in macrocytic anemias of various types.

Prompt beneficial results in Addisonian pernicious anemia treated with folic acid have been reported by various workers (160 to 164). Suggested dosage levels in general are a little higher than the 20 mg. mentioned above, although this is probably sufficient for maintenance. A few contradictory observations have been published: Castle *et al.* (165) found all the known components of the B complex, including folic acid, ineffective in treatment of pernicious anemia. Since the dosage of folic acid was only about 3 mg. a day, this is not surprising in view of the later work. Stokstad & Jukes (166) incubated parenteral liver extract with dried chicken pancreas (as a source of vitamin $B_c$ conjugase) and found the folic acid content to be about 1 mg. per cc. Parenteral liver extract did not affect the growth rate of chicks, whereas folic acid produced an effect. Not only is highly purified liver extract practically devoid of folic acid, but there is apparently no chemical similarity between the active principle in the extract and folic acid (160). Both substances appear to have similar therapeutic results. Information is not at hand to indicate any physiological relationship.

Darby *et al.* (167, 168, 169) and Spies *et al.* (170), have published results of folic acid treatment in sprue. On doses of 10 to 15 mg. daily, all patients showed improvement. Complete hematological response occurred promptly; the fatty diarrhoea improved, but more slowly. The former authors reported three cases in which the evidences of impaired fat absorption were still present after two months' treatment, although the patients showed marked clinical and hematological improvement.

A sprue-like syndrome, characterized by steatorrhoea and stomatitis, is occasionally seen in patients with dysentery, particularly after large or repeated doses of sulfonamides (171). This effect is probably due to interference with biosynthesis. Carruthers (172) studied six patients with chronic diarrhoea and anemia and concluded that in any long-standing diarrhoea a nutritional factor prolongs the production of abnormal stools. In these patients, folic acid therapy restored the stools to normal in two to five days, but they relapsed when it was discontinued. Carruthers corroborated the finding (158) that folic acid is ineffective in hypochromic anemia.

The pathological changes in the intestine in sprue have been

studied by Black *et al.* (173). They found that the percentage absorption of fat is about the same whether a high fat or low fat diet is taken. On a very low fat diet, the fat content of the feces is approximately normal. Apparently the defect in sprue is not an excretion of fat into the bowel, but failure of absorption. The constancy of percentage absorption on different levels of fat intake suggests that the defect is a failure in an enzyme system (e.g. faulty phosphorylation) rather than diminished absorptive power of the surface of the bowel wall. Both yeast and liver extract improve fat absorption, the former more markedly. The effective factor is not nicotinic acid, riboflavin, or pantothenic acid, and is probably folic acid.

Zuelzer (174) has shown that anemias with megaloblastic formation in the marrow are common in white infants. In a series of twelve infants (175) with this type of anemia, treatment with folic acid was completely effective in all but three, who died of coexisting acute infection. Folic acid was quite ineffective in infants with a normoblastic bone marrow.

Thymine (5-methyl uracil) was given in doses of 4.5 gm. or more to six patients with pernicious anemia, with good hematopoietic response (176). In doses of 15 gm. it was found effective in the anemia and steatorrhoea of tropical sprue (177). Although the hematological response was similar to that in folic acid therapy, the clinical improvement was less dramatic. This effect of thymine is of interest because of its distribution in animal tissues.

The causative role of nutritional factors in liver disease has received considerable attention and recent editorial review (178). Both necrosis and cirrhosis can be produced experimentally in animals by dietary deficiency. It seems likely that cirrhosis and yellow atrophy are due to absence of substances needed for normal metabolism, not directly to toxic agents. In the absence of sulfur-containing amino acids in the diet, a massive liver necrosis is produced. Glynn *et al.* (179) have produced liver necrosis experimentally by feeding a diet deficient in cystine. In this connection, it is of interest that Wilson *et al.* (180) have noted an improved clinical course in patients with infective hepatitis to whom 5 gm. of cysteine were administered daily. The improvement appeared to consist in a decrease in the number of relapses that occurred. Hoagland *et al.* (181) noted no improvement when methionine, choline, or crude liver extract was added to a high protein diet in

cases of infective hepatitis. Experimental evidence is lacking for a relationship between acute yellow atrophy and experimental liver necrosis. Himsworth & Glynn (182) suggest that acute yellow atrophy following an illness which ordinarily produces only transient liver damage is due to a deficiency secondary to the original disease. Eddy (183) studied a group of negro patients with severe hepatic damage as the result of carbon tetrachloride poisoning. He found a remarkable variation in the relationship between the amount of damage and the amount of exposure to carbon tetrachloride; he believed the variation to be due to nutritional factors. It is suggested that the most severe cases were a manifestation of protein deficiency. He found methionine, or a combination of cystine and choline (but neither alone) effective in treatment. Portal cirrhosis can be experimentally induced by several different types of diet either high in fat or low in lipotropic factors. The feature common to these diets is that they produce a severe and protracted fatty infiltration of the liver. Beams (184) has reported that choline and cystine, when added to a high protein, low fat diet, supplemented with yeast, produces increased improvement in patients with cirrhosis in whom the liver is enlarged, but not in those whose livers are not enlarged. He suggested that the therapy is effective in those cases which have excessive fat deposition in the liver.

Shapiro & Richards (185) demonstrated prothrombinopenia in twenty out of twenty-three cases of liver disease. When vitamin K was administered at levels of 6 to 20 mg. per day for a week the prothrombinopenia was unaltered even in instances where bromosulphophthalein retention and cephalin flocculation were normal. Restoration to a normal prothrombin level was said to indicate normal liver function.

Further investigation has been done on the effects of massive doses of vitamin D. Krestin (186) has found that single doses of 300,000 I.U. vitamin D protect infants under two years from rickets as well as, or better than, standard cod liver oil preparations. He suggests that such a dose increases the body stores of the vitamin for eight to twelve weeks; this implies that children should be given two doses per winter. Wolf *et al.* (187) found 600,000 units adequate to protect small infants from rickets. Krestin (188) found 300,000 to 600,000 units produced fair healing of infantile rickets, but the results compared unfavorably with large doses of cod liver

oil daily. None of the infants studied showed any toxic effects of the large doses. However, various investigators (189 to 192) have reported on vitamin D intoxication. The adverse effects consist of metastatic calcification and renal failure, sometimes fatal. Bauer & Freyburg (192) suggest the critical dosage level to produce toxicity is about 20,000 units per kg. body weight daily. Toxicity is more apt to develop in patients with gastro-intestinal dysfunction, and toxic symptoms have been reported on doses as small as 1000 units per kg. Jones (189) found that large doses of vitamin D administered to rats produced a degree of hypercalcemia dependent on the amount of calcium in the diet.

The change in nutritional needs in various pathological conditions has been reviewed recently (193). The most prominent change is in protein metabolism; the magnitude of this change is becoming increasingly apparent. It is estimated that after a severe burn covering 60 per cent of the body surface, 2 kg. of protein are lost in the first ten days. Both trauma and infections cause excess catabolism of protein. Following burns there is loss of nitrogenous compounds from the burned surface and in the urine (194). Experiments on burned rats (195) show that an increase in the dietary protein substantially reduces the tissue wasting which is seen in animals on a moderate or low protein intake. The urinary nitrogen loss after burning is reduced by inclusion of methionine in the diet, although other amino acids are ineffective. This suggests that after burns there is a deficiency of the one amino acid, with consequent raiding of the whole protein molecule. It is suggested that methionine be given to burned patients who are unable to take a high protein diet. Levenson *et al.* (194) suggest at least 400 gm. of protein daily for burned patients. In cases where the requirements were not met, they found that healing was delayed and death frequently ensued. A high protein diet is poorly tolerated during the shock phase after burns, but is advisable thereafter (196).

Peters (197) reported a study of two patients with severe exfoliative dermatitis. In one patient, some 40 per cent of the protein intake was being lost in the desquamated skin. Administration of 1 gm. cysteine or cystine daily produced rapid improvement in the skin lesions.

Study of the use of casein hydrolysates and amino acids has continued; this subject is further discussed in the next section. It has been found (34, 198) that mixtures of pure amino acids given

parenterally are adequate for nitrogen balance; no toxic signs are encountered unless solutions containing considerable phenylalanine are employed. Solutions up to 12 per cent concentration are tolerated subcutaneously or intravenously. Better results are obtained by giving amino acids or casein hydrolysates orally, rather than parenterally.

## Observations of Nutrition under War Conditions

Wartime food rationing has afforded a valuable opportunity for studying large groups of people whose dietary intake was better known and better controlled than was possible in years of peace. Interest was further stimulated by the opportunity to study the liberated countries, and liberated prisoners-of-war. Thus large groups of people have involuntarily been subjects of experiments which humanitarian considerations would have made impossible, if undertaken deliberately. Observations from such people have often been surprising and have led to considerable readjustment of our ideas about deficiency diseases.

The nutrition of Great Britain has been intensively studied throughout the war years. The principal changes in the diet during those years have been reviewed by Magee (199). Although the intake of meat, fish and poultry, of tomatoes and citrus fruit, of other fruits and of sugar, decreased considerably, there was a 28 per cent increase in the use of milk and milk products and a 34 per cent increase in use of vegetables. Grain products and potatoes also came into increased use. Total protein intake increased slightly, but a larger percentage was derived from vegetable sources. In the worst year, 1941, the food value of the average dietary was about the same as in the pre-war years. During that year, the people in general showed some weight loss, and growth rates of children were retarded slightly; since that time, however, the weights and heights of children have increased over the pre-war level. Correspondingly, maternal and infant death rates, tuberculosis deaths, and anemia, have all decreased below pre-war levels. Teeth of young children show a marked decrease in caries. Nutritional status has improved slightly since 1938. Provision of multivitamin supplements has been tested with large groups and found ineffective in producing any measurable beneficial effect, probably because dietary supplies were adequate.

Chattaway *et al.* (200) studied the diets of Leeds school children

in 1943–44 and found that the amounts of various nutrients obtained were below the N.R.C. Recommended Allowances and that a high percentage of the protective foods was being supplied by school-feeding. Bransby (201) studied 849 families and found that, despite rationing, the higher income group was obtaining more of the protective foods, a more varied diet, and more cooked meals. Black (202) did ascorbic acid saturation tests on a hundred adults in 1943 and found that low intakes were the result of ignorance or apathy, not poverty. These and other observations suggest that the wartime improvement in nutrition in Great Britain was largely due to the compulsory equalization of food distribution and to scientific planning of food supplies. The improvement may not persist after rationing ceases, unless economic changes are made and educational efforts are continued.

There was considerable variation in food conditions in the liberated countries. In Poland, in early 1945, the average daily caloric intake for adults was 1200 to 1500 Calories; for adolescents, 700 Calories (203). Supplementation of this ration from the black market was inconsiderable, since the total food supplies of the whole country were grossly inadequate. In Austria (204,205,206) between June and September, 1945, the official ration provided only 800 Calories daily, with very poor intakes of calcium, vitamin A, and ascorbic acid. At this time, hunger edema was appearing, and all the population showed weight loss. Extra food supplies were imported, bringing the average diet up to 1700 Calories by November. In the British Zone of Germany (204,207), in March, 1946, the standard ration was 1000 Calories; rickets and nutritional anemia were increasing and the older children showed retarded growth. Famine edema was to be found among the aged and infirm. In Jugoslavia (204) indigenous food production yielded an average of 1600 Calories daily, but this was unevenly distributed, and rickets was increasing owing to an extreme shortage of milk. In Marseilles (208), during the occupation, adults were obtaining only 60 to 70 per cent of the League of Nations standard for Calories, with low intakes of animal protein and fat. On this diet, 70 per cent of adults lost weight, and some famine edema was seen in prisons and institutions. At liberation, the people showed little evidence of specific deficiencies, but were rather thin, pale, and lethargic. Intakes for younger children were more nearly adequate than for adolescents. The children showed normal growth up to

about ten years, but thereafter growth was retarded, and a majority of adolescent girls had amenorrhoea, which was promptly cured by increased protein intake. Stuart (209) reports that about 50 per cent of the Marseilles children examined showed no sign of malnutrition. In Belgium it was found (210) that the levels of serum protein remained normal on only 15 to 25 mg. or so of animal protein daily. Although the nitrogen balance was negative in people subsisting on the official ration in 1941–42, it became positive when enough fat was added to the diet to make the caloric intake adequate. Drummond (211), summing up conditions in the liberated countries, pointed out that on intakes around 1700 Calories, although there was considerable initial weight loss, particularly in the obese, health was not impaired. He considers about 1500 Calories to be the critical level; below this, protein-deficiency anemia appears, with impaired mental application and incoordination. At a 1000 Calorie intake, famine edema, diarrhoea, and vitamin deficiencies appear, and if food intake is maintained below this level, starvation occurs.

In all these studies, the absence of stigmata of vitamin deficiencies is noteworthy. On severely restricted diets, consisting mainly of cereals and vegetables, the "classical" problems of hunger edema, weight loss, and retarded growth far outweigh vitamin deficiency diseases.

In some instances, the spotlight of wartime interest has simply brought to prominence nutrition problems that have existed for many years. Thus, the diet of Japan, which is deficient in protein, fat, and thiamine, cannot be made adequate by indigenous production (212). The loss of Manchuria has cut their soybean supplies to 40 per cent of the pre-war level, and fish are disappearing from Japanese coastal waters. Adolph (213) points out that the Chinese diet is largely vegetarian; twenty years ago only 5 per cent of their protein came from animal sources. Inclusion of green leaf vegetables in the diet would improve the protein supply. Although polished rice is deficient in the B vitamins, it is more digestible than unpolished rice; this consideration must be balanced against the vitamin supply problem. Fehily (214) points out that Chinese appear to have a lower fat tolerance than Occidentals: Chinese babies are unable to digest reconstituted whole-cream milk. Kark *et al.* (215) found that Indian soldiers ate practically no meat and received little animal protein; their average serum protein level was 5.7 gm. per 100 cc. as compared with 6.4

in Canadian and American soldiers, but the majority were fit and well, with small muscles but good muscle tone. Hemoglobin, serum ascorbic acid, urinary ascorbic acid, and riboflavin were lower in Indians than in the Occidental troops, and slightly higher than in Japanese soldiers. Folliculosis, corneal invasion, and cheilosis were fairly frequent among Indian troops.

Burger *et al.* (216) report that at liberation 5 per cent of the population of Holland was seriously undernourished. These people frequently showed gangrene of the skin and there was often a dun-colored pigmentation of the skin. No signs of vitamin deficiency were seen except slight reddening of the tip of the tongue, possibly attributable to nicotinic acid deficiency. Pulse rates and blood pressure were abnormally low. All the anemia seen was characterized by a high color index. Edge (217) studied three hundred prisoners-of-war who had been on forced marches and a starvation diet, and found that about one-third had diarrhoea (without a causative organism found), forty had edema, and many showed parasthesias of the lower limbs, with absent tendon jerks. He believes thiamine deficiency a prominent feature of starvation. Lipscomb (218) states that 60 per cent of the people in Belsen concentration camp were actually starving when the Allies took over the camp. These patients were suffering from absence of both food and water, and only 6 per cent had gross edema. Vitamin deficiency signs were strikingly absent, although normocytic anemia was common. Almost 100 per cent of the patients had diarrhoea. The weight loss varied from 30 to 55 per cent, in those well enough to stand on the scales. The average serum protein level was about 5.0 gm. per cent (219).

Keys *et al.* (220) stated that the hypoproteinemia common in Europe was mild and not closely related to the occurrence of edema. They studied thirty-four young men on a European type of famine diet (providing 49 gm. protein daily) for some months and found that the average weight loss was about 25 per cent of body weight. There was a small decline in plasma protein and edema developed. Venous pressure was 50 per cent below normal. They state that famine edema is not simply a result of hypoproteinemia, or of renal or cardiac failure.

Internees and prisoners-of-war from the Far East showed a characteristic syndrome that had not previously attracted much attention, although it had been noted in association with recognized deficiency diseases in the Orient and in Africa. This syndrome

consists of diminished visual acuity, usually with central scotomata; on examination the changes in the fundus are those of retrobulbar neuritis. The extent of visual loss varied; when it was severe, the optic discs usually showed temporal pallor (221). The visual signs often developed rather suddenly, frequently accompanied by nerve deafness and ataxic paraplegia. The changes usually did not appear until one or two years of captivity had passed; new cases often appeared rather abruptly after a dysentery epidemic (222). The cause of the syndrome is not known, although it seems certain that one or more members of the B complex are involved. Various vitamins have been tried, but in general the results of treatment have been disappointing except in very early cases. Dansey-Browning & Rich (223) believe it to be an irreversible change due to thiamine deficiency, of the same etiology as alcoholic amblyopia. They believe that vitamin A deficiency and perhaps riboflavin deficiency are contributory factors. Moore (224) points out that in its early stages nutritional retrobulbar neuritis responds to autoclaved "Marmite," so that thiamine deficiency is not likely to be the causative factor. Hobbs & Forbes (222) mention that the syndrome is frequently associated with lesions of the mouth and tongue, and with neuritic changes. They found yeast ineffective in treatment but animal protein effective. This action may be concerned with promoting intestinal synthesis of vitamins. Moore (224) states that the syndrome is seen in Nigeria, where it is confined to manioc-eaters. He believes that it occurs in groups where protein intake is inadequate, and there is a relative overconsumption of carbohydrate foods, poor in the B vitamins. If the syndrome continues without treatment for six to twelve months, it is not amenable to any treatment. Although this syndrome was not seen in Europe, a group of German prisoners-of-war in the Middle East showed a similar development of retrobulbar neuritis, nerve deafness, and ataxic changes (221). The remarkable feature in this series was the comparative rarity of other signs of vitamin B deficiency. In these patients, too, prolonged dietary treatment with a full diet, liver extract, yeast, and various synthetic B vitamins failed to effect much improvement in the neurological changes. Clarke *et al.* (225) point out that the characteristic syndrome developed in Hong Kong prisoners in almost epidemic proportions within a few months of captivity, reached a maximum, and then tended to remain stationary or

improve slightly without treatment. For this reason they feel that the condition is not a pure vitamin deficiency, but that some toxic or antivitamin principle is involved: in this case, probably in the rice which formed the great part of the diet.

Beriberi was also common in prisoners in the Far East. In Japan, Gottlieb (226) found that it did not respond well to thiamine alone, and believed it to be a multivitamin deficiency. Cullinan *et al.* (227), studying an outbreak of beriberi among rice-eating African forces, concluded that rice which had been improperly stored contained a substance which destroyed its thiamine content during cooking. Since the rice given to prisoners in Hong Kong was partially spoiled, this factor may have been operative in producing the high incidence of beriberi seen in that group. Of one hundred repatriated prisoners-of-war from Hong Kong, sixty-eight had edema and neuritis, presumably beriberi (228). Most of the Hong Kong cases were of the wet type. The Hong Kong captives had nearly as much pellagra as beriberi; glossitis and buccal ulceration were the commonest signs, and pellagra probably was a causative factor in the diarrhoea that was a frequent feature of captivity (229). Anemias, both hypochromic microcytic and macrocytic, were common (230). Harrison (231) states that the outstanding painful symptom complained of in Hong Kong was "electric feet." This symptom improved when beans, wheat bran, and rice polishings were fed but was not responsive to thiamine alone. Harrison also noted that at least 19 per cent of prisoners had a high diastolic blood pressure at some time. He believes both changes are due to spasm of blood vessel walls, since they improved when either nicotinic acid or amyl nitrite was given (both vasodilators). Another common symptom in prisoners was nocturnal diuresis, for which no explanation was found (229). In Singapore it was noted that many prisoners lost one-half to one inch in height, probably due to poor muscle tone, and absorption from intervertebral discs, joints, and heel-pads. Most of the women had amenorrhoea during captivity. Clinical vitamin A deficiency, and corneal vascularization were not seen, although scrotal dermatitis and mouth lesions, attributed to riboflavin deficiency, were seen. Repatriated American soldiers from Japan showed marked weight loss, but avitaminoses were notably lacking (75).

The question of treatment for severely malnourished persons

aroused considerable anxiety, and little knowledge was available in advance. Protein hydrolysates were expected to form a large part of the treatment of the worst cases. In practice it was found that better results were obtained by administering a high Calorie, high protein diet (216). The hydrolysates of low concentration required intravenous administration of an amount of fluid in excess of that which could be handled by an enfeebled circulation; higher concentrations of hydrolysates caused venous thrombosis (232). Concentrated serum was more satisfactory for parenteral administration. It was found that starving patients had severe anorexia and could not be persuaded to take hydrolysates by mouth. All but 5 per cent of the starving people in Belsen camp were able to take food by mouth (218). They were fed cautiously with small amounts of fluids for three to four days and the diet was then gradually increased up to 3000 Calories. The food given was high in protein. It was found that anemia, diarrhoea, and edema cleared rapidly on this regime; administration of supplemental iron or nicotinic acid was unnecessary, and effected no greater improvement. Likewise the hemorrhagic tendency noted in some patients did not yield to ascorbic acid, but disappeared when an adequate diet was given (216). In the seven weeks after the liberation of Belsen camp, the average serum protein level had risen but had not yet reached a normal level (219).

From the great mass of propaganda which was disseminated during the war years, nutrition is slowly emerging as a branch of science in which a large amount of research is needed. The war provided in Great Britain, at least, an opportunity to prove the value of a food policy based on scientific data. War, and post-war, experiences in acute deprivation showed that severe lack of food causes starvation but not necessarily vitamin deficiencies. Those experiences demonstrated that some current conceptions of requirements are very generous and also that Mitchell's adaptation explanation is sound. It would be unfortunate if the lessons to be learned from the sufferings of many persons were brushed aside and if the viewpoint in nutrition were not based on realism. It is evident that investigations are needed with regard to human requirements and with regard to methods of detecting suboptimal states. A review of recent literature leaves the impression that sound progress in nutrition can be achieved only by adherence to scientific method.

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# CAROTENOID AND INDOLIC BIOCHROMES OF ANIMALS[1]

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Among the more tangible and revealing aspects of comparative animal biochemistry are problems surrounding the various biochromes or natural pigments (45). The number of known naturally occurring colored molecules is steadily increasing, while nutritional, genetic, clinical, and pathological studies are gradually yielding important information regarding some of the physiological features of several pigment classes. But a full understanding of the biochemical significance and metabolic economy of animal biochromes is to be gained only by comprehensive researches along a highway which, although broad, is likewise long and difficult. The great pigmentary variations among animals according to species, sex, particular tissues, season, physiological condition, nutrition, hereditary background, and other factors are sufficient to suggest the scope of the field.

Because of the necessary limitation of space, and in view of the existing wealth of information concerning the carotenoids and indoles (including indigoids and melanins) this review will be devoted to certain comparative aspects of these two outstanding biochrome classes.

In the interests of breadth which must be a part of any comparative survey of this kind, references to certain of the older researches and surveys are desirable and indeed unavoidable.

## CAROTENOIDS

The carotenoids of animals commonly occur dissolved in minute oil droplets, to which they impart yellow, orange or red colors; alternately, such compounds may be conjugated with proteins to give blue, green, violet, pink, brown, gray, or other hues.

Carotenoids of several classes are stored in numerous tissues and at various concentrations by animals in the evolutionary

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scale from Protozoa to the highest vertebrates, including man. As is true among bacteria and fungi, however, there are notable exceptions throughout the animal phyla. Some invertebrates and numerous mammalian species deposit no significant traces of colored carotenoids in their tissues. The great majority of animals, however, store such pigments in their adipose tissue, eyes (e.g., as visual purple) or other photoreceptors, reproductive and other glandular organs, and in integumentary cells or products, e.g., birds' feathers.

The chemistry and nomenclature of the carotenoids are discussed amply in various works (10, 65, 118, 123, 186, 193); we shall here consider some aspects of the occurrence and disposition of carotenoids in various animal phyla.

## Protozoa

In this phylum we are bound to consider not only the true animal organisms but the flagellated phytozoans (e.g., *Euglena*, etc.) as well. Certain green euglenoid flagellates, pursuing a holophytic existence, lose their chlorophyll when deprived of light, becoming saprophytic or even holozoic, but retaining the red pigment of the "eye-spot."

Carotenoids in certain species of phytozoa give rise to the conspicuous yellow, orange, or red colors sometimes observed in snow, rain ponds, lakes, salt ponds, and patches in the sea (9, 50, 57, 106, 147). Parasitic infusorians have been observed to acquire carotenoid pigmentation by consuming the eyes of certain crustaceans (20).

## Sponges

Numerous poriferan species show brilliant orange, yellow, red, or purple pigments, whose lipophilic character was recognized by Krukenberg (82) more than sixty years ago. Qualitative chemical examination of extracts of a number of sponge species by Lönnberg (97, 98, 99) and Lönnberg and Hellström (103) revealed the carotenoid nature of the pigments.

From the red sponge *Axinella crista-galli* Karrer and Solmssen (79) have crystallized astacin, the red acidic tetraketo carotenoid derivative of astaxanthin (86) common in many crustaceans. Another red sponge, *Suberites domuncula*, was reported by Karrer & Solmssen (79) to yield a similar red carotenoid with a single ab-

sorption maximum at 500 m$\mu$ in carbon disulfide. However, Lederer (92) failed to detect such a compound in the same species; he encountered neither acidogenic carotenoids nor xanthophylls, but reported substantial amounts of two suspected hydrocarbons resembling torulene (characteristic of red torulas) and lycopene (of tomatoes) respectively. *Ficulina ficus* likewise yielded isomers or derivatives of lycopene, as well as $\alpha$- and $\beta$-carotenes, found also in *Suberites*.

Drumm & O'Connor (26) and Drumm *et al.* (27) found no astaxanthin in the red sponge *Hymeniacidon sanguinea*, but crystallized from this species both echinenone, the ketonic provitamin A from echinoids (94) and $\gamma$-carotene. Drumm and his colleagues suggest that the common presence of echinenone in both sponges and echinoids may indicate a phylogenetic relationship between these two animal groups.

From a comparative biochemical standpoint, the apparent preponderance of carotenes over xanthophylls in some sponges is quite striking, since marine algae, fishes, and the great majority of invertebrates investigated store chiefly xanthophyllic carotenoids in their tissues (52).

## Coelenterates

In this phylum, which includes the jellyfishes, sea anemones, corals, and their allies, we encounter the greatest variety and brilliance of animal pigmentation. The latest reviews on the subject have been written by Lederer (93) and by Fox & Pantin (49).

The fleshy parts of anemones and corals owe their various red, orange or yellow colors to carotenoids, while even some of the blue jellyfish, e.g., the siphonophore *Velella spirans*, acquire their striking color through the conjugation of carotenoids with protein (70, 81). The numerous papers of Lönnberg (97, 98, 99, 102) provide an extended list of coelenterates in which carotenoids were qualitatively demonstrated.

It has been established by M. & R. Abeloos-Parize (3) that the red, brown, and green varieties of *Actinia equina* derive their carotenoids originally from the diet, and that the color variants exhibit a striking specificity either in the primary selective assimilation of a particular carotenoid or in the metabolism of a common compound assimilated by both variants. A new, unstable acidogenic carotenoid, actinioerythrin, occurs in the red variety of *Actinia*

*equina*, while the green form possesses also a red-orange xanthophyll which, when conjugated with protein, provides the green color. Both red and green forms yield $\alpha$- and $\beta$-carotenes (40, 71, 88). Newly described xanthophylls, sometimes accompanied by smaller quantities of familiar carotenes, are reported to occur in *Anemonia sulcata*, *Metridium senile* (=*Actinoloba dianthus*) and *Tealia felina* (71).

An acidogenic carotenoid, closely resembling one reported by Heilbron *et al.* (71) to occur in *Tealia felina*, was discovered by Fox & Moe (47) in the small Pacific Coast anemone *Epiactis prolifera*. Fox & Pantin (48) encountered considerable assortments of carotenoids, in widely varying quantities, in the numerous color-phases of the plumose anemone *Metridium senile*. In general, red forms contain the greatest quantities (e.g., nearly 15 mg. per cent), but fewer different carotenoid compounds, while the melanistic brown varieties yield the lowest quantities, but in the greatest and most variable assortments.

While *Metridium senile* stores the carotenoid material in its own tissues, *Cribrina xanthogrammica* has been shown by Strain *et al.* (154) to owe its kinds and quantities of such pigments to the presence of symbiotic algae (zooxanthellae) living within the host.

## Worms

While the platyhelminth, nemertean, annelid, and gephyrean worms provide representatives which are richly colored, relatively little study has been devoted to the carotenoids in these phyla. Actually, hemes and melanins are encountered more frequently in the annelids and sipunculids, while the nemertean forms often exhibit integumentary carotenoids (97, 98, 99, 102, 103, 107).

Sumner & Fox (162) found about 0.36 mg. of carotene per hundred grams of fresh tissues of the blood-red hemoglobin-containing polychaete *Thoracophelia mucronata*. The carotene seemed to be a mixture of the $\alpha$- and $\beta$- isomers, while no xanthophylls or acidogenic carotenoids were encountered.

## Echinoderms

Bright carotenoid colors abound in the tissues of this group, expecially in the integument, digestive glands, and eggs of sea-stars and in the epidermis of numerous brittle stars. It is worthy of note that such carnivorous classes as these contain the highest concen-

trations of carotenoids, in contrast to the herbivorous or omnivorous echinoids and holothurians. Furthermore, oxygenated carotene derivatives, including acidogenic compounds and neutral xanthophylls or their esters predominate greatly over carotenes in the asteroid class, while certain ophiuroids store no carotenes at all. On the other hand, several species of echinoids store no acidogenic carotenoids, and yield more carotenes than xanthophylls. One species of holothurian, *Stichopus californicus*, contains very low and approximately equal quantities of carotenes and xanthophylls. The ketonic carotenoid echinenone has been observed in several species of echinoids examined, and in small quantities in the holothurian *Stichopus* but has not been detected in the asteroids save possibly in *Astropecten californicus* (51).

The sea-stars frequently manifest rich blue, purple, violet, pink, or brown colors which arise from the presence of acidogenic carotenoids conjugated with protein (51). Euler & Hellström (36) isolated one such deep blue chromoprotein from the skin of the star *Asterias rubens*. Euler, Hellström & Klussmann (37) suggested that this carotenoid-protein complex might serve the animal as a photoreceptor. Euler & Hellström (36) have separated similar carotenoids, usually bound as chromoproteins, from the integument of other stars, e.g., *Crossaster papposa*, *Solaster endica* and *Porania pulvillus*, while Karrer & Solmssen (79) and Karrer & Benz (78) recovered astacin from *Echinaster sepositus* and *Ophidiaster ophidianus*.

Species of ophiuroids (brittle stars) examined by Fox & Scheer (51) yielded relatively high concentrations of free and esterified xanthophylls and acidogenic carotenoids. Many of the pigment fractions were unique, but so chemically unstable in air as to preclude a thorough investigation.

The echinoids store major quantities of carotenoids in their gonadal and intestinal tissues. Lederer (90, 92) found $\alpha$- and $\beta$-carotenes, accompanied by echinenone, pentaxanthin and other xanthophylls, in the gonads of *Strongylocentrotus lividus*. Fox & Scheer (51) encountered large stores of carotenoids in the intestine and gonads of both male and female *Strongylocentrotus purpuratus* and *Lytechinus pictus*. In the former species the ovarian carotenoids exceeded by threefold the quantities found in the spermaries (i.e., 2.00 vs. 0.69 mg. per cent respectively); in both sexes the gonad pigments were found to be entirely carotenes. In *Lytechinus pictus*,

which contains similar concentrations of carotenoids, the spermaries yielded some fourfold as much carotene and about twice as much xanthophyll as were found in ovaries. Carotenes predominate over xanthophylls in the gonads and in most other tissues of *Lytechinus*. In contrast to the asteroids and ophiuroids, the echinoids examined possess but traces of carotenoid in the integument, but carry there instead large quantities of the quinone pigment echinochrome.

### Mollusks

Lönnberg (97, 98, 99, 102) has demonstrated carotenoids in nearly eighty molluscan species, including many pelecypods and gastropods and a few amphineuran and cephalopod forms. Ripe eggs, digestive gland and, in some species, skin and eyes yield substantial amounts of the pigments. Lederer (88, 92) and Fabre & Lederer (40) recovered a nonacidic xanthophyll, glycymerin, from the ripe gonads of the scallop *Pectunculus glycymeris*. This unique carotenoid is reported not to be invariably present in the scallop, however (92). From the red gonads and mantle of another scallop, *Pecten maximus*, was recovered an additional new xanthophyll, pectenoxanthin, accompanied by small amounts of esterified xanthophylls and β-carotene. High seasonal variations are reported in the carotenoid content of this mollusk (92). A nonacidic xanthophyll, similar to pectenoxanthin and partly conjugated with protein, was isolated from the gonads of the mussel *Volsella modiolus* by Euler *et al.* (37). Red tissues of other mollusks such as the cockle *Cardium tuberculatum* and the scallop *Pecten jacobaeus* yield various xanthophylls, while an astacin-like compound has been reported in the gastropod *Pleurobranchus elegans* (79). Astacin has been crystallized from red tissues of the scallop-like bivalve *Lima excavata* (150).

Gonads, digestive diverticulum and integumentary tissues of the California mussel *Mytilus californianus* have been found by Scheer (142) to yield a considerable variety of xanthophylls and a new acidic carotenoid mytiloxanthin, but no carotenes. Females possess higher concentrations of total pigments than do males (i.e., average values of nearly 5 mg. as compared with about 2 mg. per cent in fresh tissues). The carotenoids attain their highest concentrations in the gonads, but female somatic tissues also exceed corresponding male tissues in quantities of carotenoids. Oysters have

been reported to contain relatively rich stores of vitamin A (75).

The cephalopods seem to be generally poor in carotenoids, although the two-spotted octupus *Octopus bimaculatus* carries appreciable quantities, e.g., up to 8 mg. per cent of wet tissue, in the "liver," and actually secretes xanthophylls and acidogenic carotenoids in the black ink, in quantities up to 0.7 mg. per cent. Other tissues of this species contain no carotenoids, or only traces, the eyes containing a little (46).

Lönnberg (101) found carotenoids in the eyes of *Sepiola scandica, Rossia macrosoma* and *Eledone cirrosa*. None of the three species yielded integumentary carotenoids, but *Eledone* liver contained appreciable quantities.

Wald (180) found vitamin A and retinene in the retina of the squid *Loligo pealii*, but no traces of carotenoids in the other tissues. Similarly, Fox & Crane (46) recovered traces of carotenoids from the eyes and accessory nidimental glands of *Loligo opalescens*, but none, or only questionable traces, in other tissues. Of the carotenoids in the liver of *Octopus bimaculatus*, lutein, or a xanthophyll closely resembling it, is a prominent representative, accompanied by smaller amounts of less familiar xanthophylls, $\beta$-carotene and an unfamiliar carotene, and a unique carotenoid acid which appears following treatment with alkali. The same compounds, with the exception of carotenes but including xanthophyll esters, are secreted into the ink. Starvation brings about a disappearance of carotenoids from liver and ink. However autolysis of whole or minced liver results in but very slow loss of the pigments.

## Arthropods

*Crustaceans.*—Because of their brilliant colors, vast numbers of species and ubiquitous distribution, the arthropods have been extensively studied. Early investigations were conducted by Verne (172 to 175) on the red carotenoid material in the hypodermal chromatophores, blood, carapace, eyes, and eggs of numerous decapod crustaceans such as *Homarus, Astacus, Palinurus* and *Cancer*. Similar pigments were encountered as blue or other colored protein complexes in species of *Homarus, Astacus, Galathea, Porcellana, Hippolyte, Athanas, Palaemon, Portunus, Palinurus, Nephrops, Dromia, Carcinus* and *Pagurus*.

Basing his conclusions upon studies of carotenoids in eggs, eyes and other tissues of the copepod *Idya furcata*, Lwoff (104, 105) at

one time entertained the belief, in agreement with Verne, that at least a part of the carotenoid was synthesized by crustaceans from colorless precursors. Lwoff now shares the view, however, that zoosynthesis of carotenoids *de novo* has not been demonstrated with finality (personal communication, 1946).

Carotenoids are absorbed by *Carcinus maenas* directly from the food, and subsequently stored in the hepatopancreas. In males and nongravid females the pigments remain there, pending regular supplies thence to the hypoderm, but in laying females the storage organ becomes pale as the carotenoids are transported by the blood to the ovary (1, 41). Reserve supplies of carotenoids, persisting in the hepatopancreas even after some weeks of subsistence upon a carotinoid-free diet, are mobilized to the hypoderm and other tissues of *Carcinus* (2). Carotenoid proteins from the hepatopancreas appear also in the feces of *Carcinus* for some time after the crabs have been deprived of a dietary source of the pigments, but eventually the blood and hepatopancreas lose all carotenoid, and the feces become colorless (42, 43).

Quantitative investigations by Brown (13) have demonstrated losses in carotenoid in the shrimp *Palaemonetes vulgaris* when the animals are maintained upon white backgrounds, while similarly fed shrimps in brown or black colored surroundings maintained or even underwent apparent gains in their supplies of pigment. In this connection, it may be mentioned that accelereted losses in xanthophylls were found by Sumner & Fox (163) to occur in the greenfish *Girella nigricans*, but not in other species, e.g., *Fundulus parvipinnis* and *Gillichthys mirabilis* (162), when such animals were kept for extended periods in white containers. Such findings provide no proof, however, that carotenoids are synthesized by the animals, but suggest merely that environmental photic conditions influence in some way the rate or extent of assimilation or retention of dietary carotenoids. Egg-laying (leading to losses of pigment from the body), the chemical consumption of other stored materials with a temporary sparing of carotenoids, and finally factors such as cannibalism, resulting in potential gains of pigment by the predators, are considerations which cannot be neglected in such studies as Brown conducted with the shrimps.

The red acidogenic compound astaxanthin (3-3′-dihydroxy-4-4′-diketo-β-carotene) abounds in Crustacea, and was first isolated

as its derivative astacin (3-3′,4-4′-tetraketo-β-carotene) from the brownish black chromoprotein of the shell, the red complexes of the hypoderm and the blue-green pigment of the eggs of the crayfish *Astacus gammarus*, as well as from the eggs of the spider-crab *Maja squinado*, by Kuhn & Lederer (84) and Kuhn *et al.* (85), and from a series of other crustaceans by Fabre & Lederer (39, 40). The green astaxanthin-protein complex from the eggs of the lobster *Homarus americanus* has been shown to possess a range of color stability between pH 4 and pH 8, and to have an isoelectric point close to pH 7; its approximate molecular weight is 300,000; its absorption spectrum shows maxima at 640 mμ and 470 mμ. This "ovoverdin" may be reversibly dissociated into its constituent carotenoid and protein moieties by heating to moderate temperatures for short intervals in the presence of neutral salts, even in the absence of air (152, 153).

A blue carotenoid-protein system was encountered in the eggs of two species of goose-barnacle, *Lepas fascicularis* and *L. anatifera* (?) by Ball (7). This compound exhibits the same thermal dissociability as was observed in pigment from the green eggs of the lobster. Ball also produced a similar reversible dissociation by careful acidification of chilled systems of the chromoprotein in the presence of ammonium sulfate.

Sørensen (150) shares the general belief that crustaceans are incapable of the biosynthesis of astaxanthin *de novo*, but considers the pigment to be an oxidation product of β-carotene or of certain xanthophylls of the diet. This view seems to be the correct one, and applies in all probability to similar pigments in coelenterates, sponges, mollusks, echinoderms, and other invertebrates. The common occurrence of acidic or acidogenic carotenoids among many invertebrate animals suggests a unique ability of such organisms to carry out partial oxidation of commoner polyene molecules without actually splitting them.

*Insects.*—In view of the great numbers and prominence of insects, it is perhaps somewhat surprising to find that there has not been more attention devoted to the occurrence and metabolism of carotenoids in this colorful class.

Palmer and Knight (127) found, many years ago, that yellow carotenoids are transferred from leaves, consumed by the larvae, to the hemolymph of the potato-beetle *Leptinotarsa decemlineata*, and

hence to a predaceous hemipterous insect *Perillus bioculatus*, which consumes the eggs and sucks the golden yellow lymph from larval and adult beetles.

Carotenoids, transferred by the honeybee *Apis mellifera* to both honey and wax, are derived chiefly from the pollen which is consumed (145, 188). A number of carotenoids, including a lutein ester and $\beta$-carotene, have been isolated by Tischer (168) from propolis (bee-glue) which the insects derive from pollen. Certain species of ants reportedly deposit carotenoids in their eggs (37).

Przibram & Lederer (131) found $\alpha$- and $\beta$-carotene in the walkingstick insect *Dixippus morosus*, and carotene in the green mantis *Sphodromantis bioculata*, but not in brown variants of the same species, even if fed carotene-rich diets. In *Phyllium pulchrifolium* Przibram & Lederer detected carotene in all stages of metamorphosis, but no xanthophylls in any of the above-named species. From the red wings of *Oedipoda minuta*, however, $\beta$-carotene and a capsanthin-like xanthophyll were recovered.

The clover-eating *Colias philodice* gives rise to a dominant larval mutant which owes its green color to the presence of dietary carotenoids, accompanying the blue degradation product of chlorophyll which occurs also in the carotenoid-free blue recessive. While the green mutants fall prey to birds less frequently than do the blue ones, because of the protective coloration of the former against the green foliage, both variants are parasitized by a hemipteran insect *Apanteles flaviconchae*. The latter predators ultimately spin yellow or white silk in their cocoons, according to whether they have preyed upon the green or the blue larvae respectively (58).

Certain insects are able to subsist upon diets entirely lacking in carotenoids or vitamin A. Examples are the flour-beetle *Tenebrio molitor* (166), the clothes-moth *Tineola biseliella* (22) and the cockroach *Blatella germanica* (11, 119). Such animals yield no carotenoids from their extracted tissues.

Uda (170), Gerould (58), Manunta (108 to 111) and others have given special attention to the genetic aspects of the silkworm *Bombyx mori* and its ability to spin yellow silk, containing carotenoids, or white silk, lacking them. One gene seems to be necessary for achieving carotenoid pigmentation of the blood and serigenous gland from alimentary sources, while a separate factor controls the ability to secrete the carotenoid in the silk.

The relatively rich supplies of carotenoids in the elytra of the

lady-beetle *Coccinella septempunctatum* seem to be variable in nature depending perhaps upon the pigments derivable from this insect's consumption of aphids, which in turn receive their supplies of carotenoids from a plant host (92).

### CHORDATES

*Tunicates.*—The major contribution on the conspicuous pigments of many simple and compound ascidians is that of Lederer (89, 92), who concentrated on four species, viz, the two solitary forms *Halocynthia papillosa* (= *Cynthia papillosa*) and *Microcosmus sulcatus*, the "social tunicate" *Dendrodoa grossularia* and the compound species *Botryllus schlosseri*. From the dark red tunic and orange internal organs of *Halocynthia papillosa* were obtained rich yields of astacin, moderate quantities of a new xanthophyll, cynthiaxanthin, and traces of $\alpha$- and $\beta$-carotene. The violet-cloaked *Microcosmus sulcatus* was found to contain much free and esterified xanthophyllic material and smaller quantities of $\alpha$-carotene and echinenone. Cynthiaxanthin or zeaxanthin, lutein, and fucoxanthin were suspected among the xanthophylls.

Like *Halocynthia papillosa*, the violet-red or rose-colored social ascidian *Dendrodoa grossularia* was found to contain much astaxanthin, accompanied by smaller amounts of xanthophylls and traces of $\alpha$- and $\beta$-carotene.

The brown-red compound tunicate *Botryllus schlosseri* seemed to exhibit considerable variation in kinds and amounts of carotenoids. Lycopene and $\beta$-carotene were found, and capsanthin, capsorubin and pectenoxanthin were occasionally present. Such pigments as capsanthin and capsorubin were believed to be derived from the animals' consumption of pimento pepper wastes which were dumped into the harbor habitat.

The presence of heavily oxygenated carotenoids, e.g., astaxanthin, in numerous invertebrate species suggests a flexibility in the metabolism of dietary carotenoids which insects and vertebrates, save perhaps for some fishes, have largely lost.

*Fishes.*—The most conspicuous skin-colors in the vertebrates are undoubtedly to be found among the fishes. Numerous bright red fishes store astaxanthin or similar pigments which are probably derived from the consumption of invertebrate animals. Examples are the flesh of the salmon species *Salmo salar* (35, 38) and *Onchorhynchus nerka* (6), the scythe-fish or oar-fish *Regalecus glesne*,

whose liver yields a red oil, and *Cyclopterus lumpus*, both sexes of which seem to mobilize astaxanthin from liver to skin and flesh during the summer spawning period (143). Lederer (91, 92) encountered rich stores of astaxanthin in the red skin, gill- and mouth-mucus, iris and sclera of the marine dorado *Beryx dedactylus*. The red variety of the common goldfish *Carassius auratus*, some but not all varieties of the freshwater perch *Perca fluviatilis*, and the rock cod *Sebastes marinus* likewise yield astacin (92). The sea-devil or angler-fish *Lophius piscatorius* contains astaxanthin and taraxanthin-like xanthophylls in the liver-oil (19, 149), while the former acidogenic pigment has likewise been reported in the flesh of trout (151) and accompanying $\alpha$-carotene in the oily orange liver of the sunfish *Orthagoriscus mola* (148).

Many other species of fish contain no astaxanthin or other acidogenic carotenoids in any of their tissues. Some specimens of the freshwater perch *Perca fluviatilis*, for example, yield no astaxanthin, but traces of carotenes and three xanthophylls. One of these shows a single absorption maximum at 510 m$\mu$ in carbon disulfide, thus resembling astacin, but differing from the latter in being neutral (92).

The red fins of the pike *Esox lucius*, the yellow-spotted skin of the eel-pout *Lota lota* and the ovaries of *Eleginus navaga* contain no astaxanthin but copious other xanthophylls and traces of carotenes (92). Taraxanthin-like pigments are very commonly encountered in various tissues of fishes (44, 92), including the eyes (179), where they may perhaps serve to increase visual acuity by reducing chromatic aberration and glare or dazzle (183).

A few investigations have been made relative to the sources and metabolism of carotenoids in fishes (44, 162, 163, 164, 185). The Pacific killifish *Fundulus parvipinnis*, the greenfish or opal-eye *Girella nigricans*, the long-jawed goby *Gillichthys mirabilis*, the garibaldi or marine goldfish *Hypsypops rubicunda*, and the surf-perch *Cymatogaster aggregatus* contain in their epidermal xanthophores no carotenes or acidogenic carotenoids, but only esterified xanthophylls of the taraxanthin class.

In *Fundulus* the female transfers free xanthophyll to her ripening eggs, while the sexually mature male undergoes a conspicuous and measurable enrichment of its integumentary carotenoid. When maintained for extended periods upon white, yellow, red or black

backgrounds, these fishes adapt themselves chromatically, tending to match the color of their surroundings. But, even when kept for weeks or months under such conditions, or when maintained in total darkness for similar periods, the animals retain in their skins essentially the same quantities of carotenoids. When maintained for fourteen weeks upon basic diets to which had been added either (*a*) the taraxanthin-like skin-xanthophyll, or (*b*) carotene but no xanthophylls, *Fundulus* was found to deposit additional integumentary xanthophyll; when receiving a diet lacking carotenoids over the same interval, the fishes underwent no gain or loss in total carotenoids. This species thus seems to demonstrate an ability to oxidize carotene to xanthophyll, and to stabilize its xanthophyll stores against metabolic oxidation.

*Gillichthys mirabilis* demonstrates the same maintenance of xanthophylls as does *Fundulus*, when kept in tanks of various colors. *Girella nigricans*, however, loses its integumentary xanthophyll when kept in laboratory aquaria. Such losses have been found to occur more rapidly in specimens living on white backgrounds than in others kept in black or even in yellow containers, when all food and incident light from above are standardized.

*Cymatogaster aggregatus*, feeding upon red shrimps (*Hippolyte californiensis*), assimilate the xanthophyll from this source, but reject quantitatively the carotenes and acidogenic carotenoids, although esters of the latter are hydrolyzed in the gut. Only a certain proportion of the alimentary xanthophyll is stored in the skin of this species, where it is re-esterified and maintained at a constant level in sexually inactive specimens. Some of the xanthophyll which has been absorbed from the gut, in excess of that which may be destined for storage in the skin or elsewhere, accumulates temporarily and in the unesterified condition in the rectal tissue of the gut, to which it imparts bright orange colors.

*Amphibians.*—Carotenes and xanthophylls have been extracted from the skin of the frogs *Rana temporaria* and *R. esculenta* by van Eekelen (171) and from skin, liver, kidneys, lungs, ovaries, eggs, oviducts, testes, and fat-bodies of summer and winter frogs by Rand (133). Brunner & Stein (18) and Zechmeister & Tuzson (190) found, in agreement with Rand (133), that, although there are highly variable quantities and ratios of $\beta$-carotene and xanthophylls, such as lutein, in various tissues, the carotenoid content is

highest in the fat-body. Zechmeister & Tuzson isolated from 491 livers (1.35 kg. fresh weight) of *Rana temporaria* 8 mg. of pure $\beta$-carotene, small amounts of $\alpha$-carotene and 60 mg. of lutein and zeaxanthin combined.

Beatty (8), studying the carotenoids of the blind and nearly colorless salamander ***Proteus anguineus*** from dark underground caves of North Italy and Yugoslavia, found $\beta$-carotene and small amounts of xanthophyllic pigment, but no esters of the latter. The xanthophylls were found in the body proper (minus viscera) while the liver carotenoid was nearly all carotene.

Zechmeister (187) places the frog in a class with man as representative of animals exercising little if any selection between the hydrocarbon and alcoholic types of dietary carotenoids, in contrast to the horse, which assimilates only carotenes, the hen, in whose tissues only xanthophyllic carotenoids are to be found, and the pig, whose lipids, blood and other tissues contain no carotenoids.

*Reptiles.*—Carotenoids are present in the skins of many snakes, lizards and tortoises. although relatively few reptiles have been examined for such pigments. Carotenoids are among the several pigments involved in the mobile homochromy of color-changing lizards such as the "Florida chameleon," actually an iguanid lizard, *Anolis carolinensis*, discussed by von Geldern (178), and an African species of chameleon shown by Manunta (110) to contain some 2 mg. of xanthophylls per hundred grams of skin, accompanied by much less free xanthophyll and minor quantities of carotene. The eggs, however, yield much free xanthophyll of the lutein class, but no xanthophyll esters and mere traces of carotene. The liver is reported to be richest of all tissues in carotenoids, containing about 10 mg. per cent; half of such pigments appear to be free xanthpohylls, a third carotenes, and the final fifth xanthophyll esters.

A carotenoid resembling $\gamma$-carotene (or rubixanthin or gazaniaxanthin) and another compound not unlike an ester of taraxanthin have been recovered from the integument of the Japanese turtle *Chrysemys scripta elegans;* an additional xanthophyll was found in the viscera (92). From the skin of *Chrysemys* terrapins in this country, Kritzler (80) extracted considerable quantities of a red xanthophyll which is distinct from lutein and shows absorption maxima in carbon disulfide at 505, 475 and 450 m$\mu$.

The present writer has extracted from the hard, waxy, corn-

grain-shaped, yellow-brown kernels from the femoral pores of the spiny-tailed iguana *Ctenosaura acanthura*(?) an esterified xanthophyll resembling taraxanthin, doubtless derived from the vegetable diet of this reptile.

*Birds.*—Concerning the carotenoids of the avian class there exists a very extensive literature, so that only certain outstanding contributions can be considered. Birds contain carotenoids in their liver, eyes, adipose tissues, serum, skin, eggs, and frequently in feather materials.

Palmer and his colleagues (125) demonstrated many years ago the fact that the yellow fat, skin and egg-yolk of the White Leghorn fowl are colored by dietary xanthophylls but contain essentially no carotenes. However, hens require vitamin A, and manufacture this from dietary carotene, subsequently storing part of the vitamin in the eggs (4). During the laying season, the xanthophylls are deposited in the egg-yolks instead of in the skin of legs and other areas. It has been demonstrated by numerous workers that various dietary xanthophyllic carotenoids, notably capsanthin from red peppers and zeaxanthin from yellow corn, are stored in skin and in yolk (14 to 17, 72, 74, 121, 169).

Carotenoids have been found in the iris of several breeds of domestic fowl (73), and in the retina as well, chicken retinal carotenoids including a lutein-like xanthophyll, a yellow-green carotene resembling sarcinene, and astaxanthin (181, 182).

Lönnberg (96, 100) found carotenoids in the eyes of twenty-two species of wild birds belonging to widely different orders, and in the skin of feet, bill, or face of fifteen other species.

The amber-colored oil stored in the proventriculus of the pelagic, surface-feeding fulmar petrel *Fulmarus glacialis* has been reported to contain no free or esterified xanthophylls or acidic carotenoids, but carotenes accompanied by rich stores of vitamins A and D (139). If such an interesting biochemical partition is indeed a fact, the oil and its contained carotenoid material must represent a secretion by the bird, rather than an undigested residue. Even so, it is difficult to visualize how the oil should fail to extract some of the carotenoid material from fresh food swallowed by the bird, and thus accumulate alcoholic carotenoids.

Certain birds may effect conversion of common alimentary carotenoids to colored compounds of altered constitution. The canary *Serinus canaria canaria*, for example, has been shown by

Brockmann & Völker (12) to store lutein itself in the liver, fat and egg-yolk, but to transform the pigment into a new, taraxanthin-like xanthophyll before secreting it into the new yellow feathers. Zeaxanthin is assimilated and stored by the same species, partly altered and partly unchanged, in yolk and feathers, while violaxanthin is altered in the digestive tract, but is not assimilated in any tissues or in feathers. Dietary $\beta$-carotene and lycopene were reported not to have been stored as such in tissues or in feathers.

Astaxanthin or a similar carotenoid has been encountered in the egg-yolk of the laughing gull *Larus ridibundus* and of the stork *Ciconia ciconia*, unaccompanied by any traces of carotene or lutein (12). Astaxanthin has also been demonstrated in flamingo fat (112).

Test (167) has investigated the carotenoids in the scarlet feathers of the woodpecker *Colaptes cafer*, the yellow plumes of the related species *Colaptes auratus*, and the intermediately orange-colored plumage of a suspected hybrid between the former two species. In general, three types of carotenoids prevailed in the three forms studied: (a) yellow xanthophylls, free and, to a minor extent, esterified, (*b*) $\alpha$-carotene and probable isomers of this, and (*c*) unknown red, neutral epiphasic and hypophasic carotenoids exhibiting single absorption maxima. The feathers of *C. auratus* owe their yellow color principally to the presence of taraxanthin-like xanthophylls and traces of carotenes, notably the $\alpha$-isomer. The scarlet hues of *C. cafer* are produced chiefly by the unique red, spectroscopically single-banded carotenoids, accompanied by some yellow xanthophylls and orange carotenes. *C. cafer* possesses a greater number of plumage carotenoids than does *C. auratus*. The predominating red color of the former species results from the presence of additional red carotenoids rather than from an actual replacement of the yellow type. Intermediately colored feathers from the orange, suspected *cafer-auratus*, hybrids contain all three classes of carotenoids encountered in the other species, but the red carotenoids in the case of the "hybrid" constitute a smaller proportion of the total than in *C. cafer*.

It has been found by Kritzler (80) that the display plumages of nuptially colored male bishop birds (*Euplectes* spp.) are rich in several carotenoids, while the henny eclipse feathers, to which the males revert after the postnuptial moult, are nearly devoid of this class of pigment. From the plumes of wild *Euplectes franciscanus*,

*E. orix* and *E. nigroventris*, and from the head-feathers of the woodpecker *Melanerpes erythrocephalus*, Kritzler extracted two unfamiliar nonacidic red carotenoids and a yellow xanthophyll resembling lutein. Notwithstanding the maintenance of postnuptial males, showing their eclipse plumage, upon carotenoid-free diets for some three months, Kritzler found rich stores of residual carotenoids in liver and body-fat. When injected with pregnant mare serum, *Euplectes afra* and *E. nigroventris* mobilized such stored carotenoid pigment to new display feathers which grew in replacement of plucked henny plumage.

There is manifestly a high degree of flexibility in the complex carotenoid metabolism of the avian class. Extensive investigations into the comparative biochemical aspects of this field are clearly indicated as important future undertakings.

*Mammals.*—While horses and cattle, typical herbivores, store carotenes in their body-fat, liver, adrenals, corpus luteum, serum, and milk, and while man, an outstanding omnivore, carries an assortment of carotenoids in the same tissues and secretions, depending on the diet, nevertheless mammals as a class are relatively poor in this type of biochrome.

The chief carotenoid in the tissues and milk-fat of cattle is $\beta$-carotene (126), which may be accompanied by traces of $\alpha$-carotene, lycopene, lutein, and cryptoxanthin (60, 61, 62). Whole ovaries of cattle are four times as rich in carotene as are testes [e.g., 0.3 mg. as compared with only 0.08 mg. per cent (187)], while the corpus luteum and corpus rubrum of the ovary may yield quantities amounting to 6 mg. and 120 mg. per cent respectively (83). Cows' plasma may contain from three to fivefold the amounts of carotene found in bulls' plasma (60), thus reflecting the greater metabolic turnover of carotene in the female (146). Vitamin A and carotenoid pigments have been obtained from the yellow patches of olfactory tissue in the upper part of the nasal cavity of cattle (120).

The blood of sheep, an antelope, and a deer were found to contain lower concentrations of carotene and vitamin A than did that of a range cow (128), while, in India, buffalo butter-fat has been found to involve only about one-tenth the concentration of carotene present in cows' butter, i.e., 0.02 to 0.035 mg. as contrasted with 0.2 to 0.57 mg. per cent respectively (6a).

The horse has been established as a typical carotene-assimi-

lating, xanthophyll-rejecting animal, the elimination of xanthophylls occurring before such compounds can enter the general circulation through the intestinal mucosa (188, 189, 192).

Although swine consume carotenoid-rich food and require vitamin A in their ordinary and reproductive metabolism (69), they probably store the least carotenoid material of all mammals so far investigated. Even their adipose tissues contain less than 0.1 mg. per kg., following an extended diet very rich in such pigments. The liver may yield from one-fifth to one-tenth of the above quantity (191).

Astacin may be recovered in considerable quantities from the liver oil and feces of whales, which derive this carotenoid from consuming vast quantities of "krill" or small crustaceans (28, 144).

Pease (129) and Willimott (184) encountered a recessive factor in the rabbit *Lepus cuniculus* which permits the deposition of xanthophyll in the adipose tissues of certain strains of this normally "white-fatted" animal. Willimott suggested that the dominance of the "white-fatted" variant might be due to the presence of a thermolabile oxidative system in the liver, the recessive "yellow-fatted" animals lacking such an enzyme, and therefore storing rather than destroying the dietary xanthophyll.

### Sediments

The high proportion of carotenes relative to xanthophylls in certain muds of the ocean floor, in contrast to the reverse condition which prevails in the green plants and the suspended detritus of the sea, may be due in considerable part to the xanthophyll-assimilating, carotene-rejecting propensities of the majority of marine animal species and in part to the biochemical reducing properties of microorganisms (52).

## INDOLIC PIGMENTS

Indolic pigments, containing the familiar indole or phenopyrrole nucleus, are among the ultimate catabolic degradation products of the amino acids tyrosine, phenylalanine, and tryptophane. In this class of biochrome are included the blue, red, or purple indigoids and the various more complex yellowish, ruddy, brown, or black melanins.

## Indigoids

Indican, or potassium indoxyl sulfate, may be detected in the urine, notably that of herbivores such as horses and cattle. It may also occur in relatively large amounts in human cases of hepatic carcinoma. It is demonstrated by mild oxidation in acidic solution to yield the blue, chloroform-extractable compound indigo. Indigo blue itself, likewise indigo red, are encountered in microgranular form in some pathological urines.

Purple secretions of certain gastropod mollusks, e.g., *Mitra, Murex* and *Purpurus*, have been known since very ancient times, and the 6-6′-dibromindigo which constitutes the chief pigment has been employed as a fabric dye. In *Purpurus lapillus* the pigment arises or accumulates in the so-called adrectal or hypobranchial gland, a pale yellow strip of tissue lying along the rectal segment between this and the gill, and attached near the end of the gut (29 to 34, 95).

The simplest indole pigment is the red 5,6-quinone of 2,3-dihydroxyindole-2-carboxylic acid. It constitutes an intermediate link in the formation of melanin (134), and occurs also in the integument of the marine annelid worm *Halla parthenopoea costa*, whence it derives its common name of hallachrome. It is easily reducible and has been considered by Friedheim (55, 56) to be an accessory respiratory catalyst, notably in the oxygen consumption of unfertilized sea-urchin eggs and mammalian erythrocytes. Confirmatory investigations of this singular quinone will be of interest and must precede any serious consideration of Friedheim's theory as to its physiological role.

## Melanins

These biochromes are undoubtedly the most widely distributed of all animal pigments, and give rise to the various shades of black, gray, brown, ruddy, tawny, and Tyndall blue. Different hues arise from the fact that the responsible melanin may exist in various stages of oxidation, in different degrees of physical aggregation, and often in diverse relationships with other pigments. Widely different animal phyla share the common biochemical property of catabolically deriving melanin through enzymic oxidation of tyrosine or related phenolic compounds.

Various colored melanins are present in the ectoderm of certain coelenterates, e.g., dark variants of the plumose anemone *Metridium senile* (48), in the integument of some holothurians and ophiuroid echinoderms (174) and flatworms (21), in the epidermal melanophores of numerous reptiles, amphibians, fishes, and cephalopods, and notably in the specialized anal gland (ink-sac) of the latter. The ink-gland was described long ago by Gessard (59), who also demonstrated the enzyme tyrosinase in melanin-free glandular tissue from fresh animals, as well as in the dried *Sepia* sacs of commerce.

Among vertebrates, melanins are encountered not only in dermal and epidermal tissues, but also in such integumentary products as hair, feathers, and scales. Internal connective or other membranous tissues, e.g., choroid, peritoneum, pia mater etc., of numerous fishes, amphibians, reptiles, and birds likewise contain black melanin.

The onset of melanization in the integument of insects seems to be associated with any of several metamorphic, stages, e.g., the newly hatched larvae of the cockroach *Phyllodromia germanica* (130), the pupating larva of the fly *Musca carnaria* (25), and in the developing caddis-fly *Limnophilus flavicornis* at the end of the larval period and in the late nymphal stage, but not in the adult (138). The integument of the developing "meal-worm" beetle *Tenebrio molitor* is heavily invested with melanin soon after the process of shedding from the pupal stage has been completed. Pupae of the locust *Tibicen septendecem* show a rapid cuticular melanization on emergence from their seventeen-year residence underground (63, 64).

Lea (87) claims that ascorbic acid, an easily oxidizable compound, inhibits the enzymic oxidation of tyrosine to melanin. He also demonstrated an acceleration of melanogenesis following depression of the normal physiological sodium chloride level.

Fresh human epidermis has been found by Rothman *et al.* (141) to contain water-soluble sulfhydryl compounds, absent in the corium, which inhibit melanogenesis in tyrosine-tyrosinase systems. Rothman and his colleagues suggest that the inhibitory SH compounds may be oxidized or otherwise altered by such melanogenic stimuli as sunshine, x-rays, etc., whereupon the enzymic "tanning" process takes place readily.

Physiological attributes of melanized insect-cuticles have been

suggested as follows by Kalmus (76, 77): (*a*) increased protection against short-waved injurious light rays, (*b*) increased rate of heat exchange, (*c*) enhanced toughness or mechanical resistance, (*d*) decreased wettability, and (*e*) protection from desiccation.

The Silky cock, bearing white feathers but black skin, mesenteries, periosteum, pia mater, and connective tissues, transmits this melanistic pattern, when bred to a melanin-free hen, only to his daughters (132).

According to Rawles (135), melanogenic skin may be grafted from embryonic robins to the wing of the developing White Leghorn chick embryo, resulting in temporary melanization of the down growing from the transplant in the hatched host. The robin's melanophores, having migrated from the graft into the host's feather-germs, express the ruddy color until moulting, the graft thereafter bearing host-colored (white) feathers.

It has been demonstrated by Vilter (176, 177) that axolotls (*Amblystoma mexicanus*) generate more melanin and greater numbers of melanophores when living upon black backgrounds than when maintained in white surroundings. Similar observations have applied to frogs (*Rana temporaria*) by Dawes (23, 24) and to fishes, e.g., the Atlantic killifish *Fundulus heteroclitus* by Odiorne (124), the guppy *Lebistes reticulatus* by Sumner & Wells (165), the long-jawed goby *Gillichthys mirabilis*, the mosquito-fish *Gambusia patruelis*, and the greenfish *Girella nigricans* by Sumner and his colleagues (159, 160, 161).

Sumner (156, 157, 158) provided incontrovertible experimental evidence for the survival value of melanistic chromatic adaptation of fishes in the direction of concealment against attack by various predators.

Red hair from the human head contains, according to Rothman & Flesch (140) a red, iron-containing, acid-soluble pigment not found in other human or nonhuman hair. The same pigment, or a closely similar one, has been isolated by Nickerson (122) from red poultry feathers, and has been found to be derivable from the oxidation of black melanin.

## Schemochromes

While the blue colors of numerous coelenterates, nudibranch mollusks, crustaceans, echinoderms, and other invertebrates are due to the presence of acidogenic carotenoids conjugated with

protein, equally arresting blue hues, occurring in the skin of numerous fishes, reptiles, some primates and the skin and feathers of many birds, arise from a very different principle. It involves reflection of the scattered short-waved (blue and violet) fractions of incident light by very fine particulate bodies of mean diameter lying between about 0.4 and $0.7\mu$, while the longer-waved components (yellow, orange, and red rays) penetrate the layers containing these light-scattering inhomogenieties and are absorbed by deposits of melanin situated beneath. Without the underlying absorbing screen of melanin, the blue colors would not appear (113, 114). These so-called "schemochromic" blues of Tyndall scattering are observable in the blue sky, in blue smoke, which casts a complementary reddish shadow, and in various opalescent systems, such as agar-gel, placed against a black surface.

Examples of Tyndall blues in the biological realm could be multiplied at great length, but may be confined to a very few for present purposes, e.g., the brilliant blue spots on the skin of the young garibaldi *Hypsypops rubicunda*, in the tail and belly of the blue-tailed skink *Eumeces skiltonianus*, in the facial and neck-skin of such birds as the turkey, guinea-fowl, and double-wattled cassowary *Casuarius galateus*, in the muzzle, scrotum and buttocks of such primates as the West African drill *Cynocephalus leucophaeus* and the mandrill *Papio sphinx*, and in the blue eyes of man, kittens, and some monkeys.

The blue colors of feathers arise from the presence of countless minute air-vesicles within the so-called box-cells overlying deposits of melanin in the barbs (53, 54, 66, 67, 68, 113). Replacement of the air in such a scattering system, by a foreign fluid, e.g., phenol, whose refractive index approaches that of feather-keratin (1.54) causes a complete discharge of the blue color, which is readily restored by leaching out the foreign fluid. Destruction of the melanin layer by oxidation with hydrogen peroxide extinguishes the Tyndall effect, but this may be restored by painting the underside of the feather-barbs with India ink.

Countless manifestations of brilliant iridescence and "metallic" colors are present in insects' cuticles, feathers of peacocks, hummingbirds, etc., and arise from the interference of reflected light-waves by very thin laminations, backed beneath by deposits

of melanin, which absorb the penetrating rays, thus rendering the reflected portion the more pure in its series of Newtonian colors (5, 114 to 117, 136, 137, 155). Removal of the melanin layer greatly diminishes the visible iridescence, which may be restored by substituting a black paint, such as India ink.

In black, Tyndall blue or iridescent feathers, the melanin involved is still to be regarded as but a catabolic by-product, discharged by integumentary cells.

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# THE NITROGENOUS CONSTITUENTS OF PLANTS

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This subject has been reviewed by Nightingale (1) and by Wood (2). Reference has also been made to nitrogen metabolism in earlier sections on mineral nutrition (3,4). The last review by Wood dealt mainly with protein regulation and with the evidence for the amino acid hypothesis of protein synthesis. To quote Vickery *et al.* (5), this hypothesis is "the simplest and most straightforward view of the problem and one that is widely, although not universally held."

## Nitrogenous Compounds *in vivo*

Of all the biochemical constituents of cells nitrogenous compounds should be the most closely integrated with vital activity. Nevertheless the major processes of plant metabolism and nutrition—respiration, photosynthesis, absorption and accumulation of salts, intake of water—were for long treated as discrete processes. In the modern view, through the use of $N^{15}$, concomitant synthesis and breakdown of nitrogen compounds is now regarded as a characteristic of life (6). The difficulty and the challenge of nitrogen metabolism is that it is not enough to explain the path of synthesis or the reactions of nitrogen compounds per se: one should know their interrelation with other physiological processes.

Spoehr & McGee (7) pointed to the role of amino acids in the respiration of leaves and the response of plant respiration to nitrate supply is now familiar. Plant physiologists were, however, slow to recognize that the processes of respiration and nitrogen metabolism, converging upon or rather interlocking at specific intermediates (e.g., keto acids), are interdependent. No longer,

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however, can respiration be treated as though it concerned carbohydrate breakdown alone (8, 10 to 14). It is the interrelations between nitrogen metabolism in plants and other vital processes which constitute a salient problem today.

## The Varied Potentiality for Growth and Protein Synthesis

The parallelism between growth and protein synthesis is evident enough for the uniform system of cells to be found in a culture of *Chlorella* (15). In the exponential phase of growth, when each cell grows at the maximum rate, protein synthesis is high; thereafter it declines sharply and becomes negligible when growth ceases. The ratio of nitrate to carbohydrate assimilated by monocotyledon leaves changes with age (16). In first leaves (lamina 11 cms.) on wheat plants twelve to fourteen days old the C/N ratio was 4.6; second leaves (lamina 20 cms. and still expanding) showed a C/N ratio of 17.6. The mature leaf synthesizes sugar and protein (which must be storage protein): in the still expanding leaf protein production makes new cells and stimulates fixation of carbohydrate as nitrogen-free building units of protoplasm and of cell walls. The region of synthesis in the monocotyledon leaf is the basal intercalary growing region and the extending region immediately above [(17), p. 324]. Pearsall (*loc. cit.*, p. 326) distinguishes two broad phases in the development of a leaf—first, it is predominantly engaged in protein (i.e., protoplasmic) synthesis; second, predominantly in photosynthesis (i.e., carbohydrate synthesis).

Similarly the tissue of the potato tuber is inherently capable of growth and protein synthesis. For a variety of reasons this capacity is not exercised in the mature intact tuber: the cells at the surface of a cut slice, however, may both grow and synthesize. Placed in moist air, they regenerate a new periderm. In aerated water or salt solution they gain in water, salt, and protein. Under the latter conditions and in a manner related to oxygen tension respiration and sugar content increase, tissue oxidations occur and the cells proceed to exercise practically all the vital functions of which parenchyma are capable [Steward *et al.* (13), p. 425].[2]

Cells tissues and organs in the plant body differ greatly in

[2] Points of contact between auxins and protein metabolism are to be expected (18, 19) in systems in which growth and protein synthesis go hand in hand.

their capacity for protein synthesis. Stuart & Appleman (20) showed that as uniform an organ as the potato tuber has lower protein nitrogen and higher soluble nitrogen in the medulla than in the cortex and the buds, with the adjacent cortical tissue, are even richer in protein nitrogen. Leaf, stem, and root, even of the same plant, may have different levels of protein and different proportions of protein nitrogen to soluble nitrogen. Green leaves have long been recognized as prime organs of protein synthesis, especially when attached and exposed to light, and here the relation between protein nitrogen and soluble nitrogen is strongly directed toward synthesis. In the onion leaf the bulk of the nitrogen is protein, especially in the young leaves. In the onion bulb more of it is in soluble form (21). In vegetative potato plants the leaf is the only organ (other than the old tuber being depleted of its soluble reserves) to contain its total nitrogen preferentially in the form of protein nitrogen (22). This property is not only a property of the attached green leaf in the light, but it also involves the source of nitrogen: as shown again by Vickery *et al.* (5) who have studied the mobilization of the nitrogen reserves stored in the bulb of *Narcissus poeticus.* When supplied with nitrate the protein nitrogen as a percentage of total nitrogen in the leaf in the light was 51.7; in the dark it was 36.4. Starved of external nitrogen the bulbs produced leaves in the light in which 60 per cent of their total nitrogen was protein nitrogen in contrast to 44 per cent in the leaves grown in the dark. With ammonia as an external source of nitrogen, however, these differences between the light and the dark grown leaves were minimized (48.7 per cent of total nitrogen was protein nitrogen in the light; 39.4 per cent in the dark). In corn (*Zea mays*) Viets *et al.* (59) found that, despite increased amino nitrogen in response to ammonia nutrition and a plentiful supply of carbohydrate, the synthesis of protein nitrogen was limited by more decisive factors. Both light and a nitrate source of nitrogen, therefore, foster protein synthesis in these leaves.

Both in cells and organs differentiation and maturity are accompanied by changing protein synthesis. In some fruits the phase of cell multiplication and synthesis of nitrogen compounds is short (24,25). The eventual loss of the capacity of cells for protein synthesis may be a graduated process. Pearsall & Billimoria (17) quote a case where young leaves of *Pelargonium* could syn-

thesize from an external source of asparagine but not from ammonium nitrate. Viets (23) showed that nitrogen-starved whole corn plants accumulated much soluble nitrogen when supplied ammonium salts but did not synthesize protein appreciably. Vickery *et al.* (5) showed that whereas attached Narcissus leaves synthesized protein using both stored reserves and supplied nitrogen, the detached leaves, free from the stimuli and interrelations of the intact plant body, did not. Indeed, the study of Vickery *et al.* on *Narcissus poeticus* leads back to conclusions which involve growth. They show how detached leaves undergo protein hydrolysis, amide and eventually ammonia accumulation leading to moribund changes, and that it was not possible to eliminate these trends by nutrition (sugar and nitrate) alone.

It is the somewhat general experience that excised leaves undergo a protein breakdown which is usually faster in the dark than in the light [Vickery *et al.* (26)]. Thus Vickery *et al.* (5) conclude that

> detached leaf experiments furnish a picture of the disintegration of the chemical systems of the cells when the controls upon these delicately adjusted relationships are removed—and these controls play their role effectively only when the organism is intact and—must have their origin in other tissues than the leaves.

In the intact shoot the leaf is subject to the translocation of its stored nitrogen compounds to the growing regions [e.g., flowering shoots, see Pearsall & Billimoria (17)]. This may account for a somewhat vicarious protein synthesis in relatively old leaves and for gradients of protein nitrogen content which may seem to be reversed, e.g., higher protein nitrogen in older leaves probably due to preferential removal of nitrogen from the more active younger leaves.

Some mature cells can return to active growth and synthesis. The potato tuber has a lower percentage of its total nitrogen in the form of protein than the leaf [Street *et al.* (22)] but as they return to a more actively metabolizing, more meristematic condition, the surface cells of the tuber will synthesize protein nitrogen at the expense of the soluble nitrogen reserves. Treatments, however, which inhibit the ability of the cells to grow also inhibit their protein synthesis (14).

Thus plant tissue systems, even of the same plant, have different ability to synthesize protein which goes hand in hand with their growth and development.

## Forms of Organic Nitrogen—The Protein Nitrogen Fraction

Inadequate methods still limit the analysis of some forms of organic nitrogen. Incomplete knowledge of plant proteins is evident from the accepted designation protein-nitrogen to cover what is clearly a heterogeneous fraction. This fraction comprises the structural proteins of the cytoplasm and the nucleus, cytoplasmic inclusions like mitochondria and plastids, together with storage proteins which may be dissolved as globulins in the cell sap or deposited in special tissues (e.g., of seeds) as crystals or granules.

Vickery (27) has reviewed the plant proteins and Chibnall (28) rediscussed the problem of protein structure giving data for some common proteins. The best known plant proteins are still storage proteins of the seeds of certain important crop plants. Only recently has it been possible to obtain representative samples of the whole proteins of leaves (29), of chloroplast protein, and composite mixtures of cytoplasmic proteins and vacuolar proteins from leaves. As Lugg (30) emphasizes, the leaf proteins so far examined are to be regarded as mixtures not as yet resolved into their individual components and, furthermore, their amino acid analyses are incomplete and their physical properties but little known. Wildman & Bonner (31) have recently reported the fractionation of spinach leaf proteins into an electrophoretically homogeneous fraction which yields auxin on hydrolysis and a second fraction, in which various enzymes have been identified, and in which subfractions may be recognized by electrophoresis.

The proteins of meristematic cells await biochemical investigation. The cytoplasm consists [Guillermond (32)] of the particulate and self-reproducing mitochondria and plastids, as well as other minute particulate inclusions (microsomes) in the ground mass of protoplasm. The analysis of chloroplast protein and the microchemical reactions of leucoplasts and mitochondria indicate the presence of lipoprotein in these structures.

The biochemistry of proteins is as yet on a too macroscopic scale for these problems of cytology. Certain investigations on animal cells, however, suggestively point the way. The cytoplasmic proteins may be separated [Banga *et al.* (33)] into fractions soluble in salts (globular proteins) and insoluble in salts (fibrillar proteins held to be the more solid structural edifice of protoplasm). Even the latter fraction is further separable into fractions resem-

bling plant viruses and myosin respectively. Controlled centrifuging [Claude (34)] separates the particulate inclusions (mitochondria and microsomes) from the transparent hyaloplasm. Controlled centrifuging and fractionation with solvents [Hoerr (35)] has been applied with some success to different cellular protein fractions. Bensley (36) pictures the cytoplasm as composed of: (*a*) a particulate fraction (mitochondria; microsomes) containing protein, nucleoprotein, flavoprotein as well as a variety of fatty compounds; (*b*) the structural fibrillar proteins of the cytoplasm of which plasmosin and ellipsin are recognized: the former containing nucleic acid and both associated with lipoid; and (*c*) a fluid phase containing globular and flavoproteins but devoid of lipoid material. Further investigations to shed light on the proteins of plant meristematic cells are clearly desirable.

The predominance of nucleoproteins (37,38) in chromosomes and possibly other cytoplasmic inclusions which are conspicuously endowed as autonomous, self-perpetuating bodies leads one naturally to identify the self-reproduction of the organ with the autonomous manufacture of its substance. The resemblance between the process of nucleoprotein synthesis and spontaneous virus multiplication (39 to 42) has, in fact, been noted.

The relative constancy in the ratios of basic amino acids (43,44,45) in keratin and serum proteins (46,48) lead to the idea (47) that the tissue proteins are built up around a nucleus—an "Anlage"—of relatively fixed proportions of arginine, histidine and lysine on which additional amino acid residues are grouped. Alcock (49) saw the chemical composition of a primitive or more universal protein reflected in the most frequently occurring percentages of their constituent amino acids.

Thus it is not hard to conceive, though it may be impossible to prove, that protein synthesis (especially of the fibrous or structural proteins of the protoplasm) may not proceed, bit by bit, on the classical amino acid hypothesis but rather, "in one operation" from those simple constituents which fit the basic pattern, whether these be only sugars and nitrate or ammonia, or some more complex preformed organic molecules.

Indeed, there may be different synthetic mechanisms for different protein fractions. Whereas the globular proteins dissolved in the fluid phases of protoplasm and the more solid storage proteins

may be manufactured *in vivo* by mechanisms which involve proteinases, along the lines of the more conventional views of amino acid condensation, there may yet be in growing cells a different mechanism for synthesizing the proteins of the organized living system itself, a mechanism characterized more by the ability of a synthesizing surface to produce a constant facsimile of itself.

The outstanding recent contributions of biochemical genetics are relevant here. The work of Beadle and his school (50,51,52) shows that protein synthesis is subject to genetic control. There are strains of the fungus *Neurospora* which grow only if supplied with preformed amino acid moieties of the protein molecule. By suitable choice of the conditions and of strains of bacteria the bioassay of almost any of the essential amino acids is now possible (52a).

At first sight these remarkable results may seem to support the amino acid hypothesis of protein synthesis: a particular strain may lack the enzyme complement to synthesize a particular amino acid and the complete ability to synthesize protein may require the combined action of a large number of specific enzymes each controlled by the action of specific genes.[3]

The genetical work on organic nitrogen nutrition and assay of

[3] It is a difficult question how far it is permissible to generalize from the abnormal course of synthesis for particular strains limited by lack of specific factors to the normal course in cells which are not so limited and which can even use inorganic nitrate. The property of mutants requiring a particular amino acid may well be a lack or disorientation of that part of the protoplasmic surface or "template" against, or by which, the proteins of growth are synthesized so that it is feasible that some preformed larger unit, like a single amino acid, might then be needed to fit into the final molecular pattern. This does not necessarily presuppose, however, that in the normal strain the course of synthesis passes through the molecule in question. Doermann (53, 54) found "lysine-requiring" strains of *Neurospora* were inhibited by arginine though the normal organism was not, and explained these results in ways which did not presuppose that the normal strain separately synthesized lysine. Hydroxyproline, though a common constituent of proteins, suppresses the growth of certain dermatophytic fungi in contrast to a variety of other organisms drawn from various groups (55). Robbins & McVeigh's comment might well be more generalized as follows—"it is clear that the relation of amino acids to growth—cannot be covered by a simple generalization. Some of them serve as sources of nitrogen, some are unavailable, some are inhibitory and their effects when two or more are present together depend upon which amino acids are used."

amino acids focusses attention on further inherited limitations which enter into the normal relation between growth and protein synthesis in heterotrophic organisms. The growing cells of autotrophic plants synthesize proteins which are part of the vital system, rather than storage substances, by a process which seems to conform much more to an autonomous self-reproduction analogous to, if not causally determining, the self-reproduction of the living inclusions in the cell. If this be so, the analysis of the amino acid hypothesis [Wood (2)] in which protein synthesis is held to respond to amino acid concentrations in cells and to be subject at least to "steady state" conditions seems labored: it may well be applicable only to cells no longer able to grow and to the elaboration therein of physiologically inactive storage products.

The total protein content of metabolizing plant systems is commonly expressed as the protein-nitrogen content: usually the difference between the total-nitrogen of the tissue and the nitrogen extractable by hot water at 80°C. or by hot alcohol, sometimes followed by hot water. Hanson *et al.* (56) and Wood *et al.* (57) obtained a separate analytical figure for chloroplast protein nitrogen. That present techniques do not permit other protein fractions to be determined indicates both a virgin field for study and an obvious limitation in present understanding of protein metabolism.

## Forms of Organic Nitrogen—The Soluble Nitrogen Fractions

A variety of nitrogen compounds are extractable by hot alcohol. The major forms of nitrogen of which the soluble nitrogen is composed fall into the fractions amide nitrogen; amino nitrogen; and the "other or residual nitrogen." Residual nitrogen may often be a large part of the nonprotein organic nitrogen and may even exceed the sum of the amide and amino nitrogen (58,59,60), and is therefore a confession of inability to resolve and estimate the components of the nonprotein nitrogen.

*Amide-Nitrogen.*—The fractionation of the amide moiety of the soluble nitrogen into the components glutamine and asparagine is still not simple though the indirect methods for the separate determination of glutamine and asparagine amide nitrogen can, in selected cases, be confirmed by the laborious procedure of isolation of biochemically pure products. [See Steward & Street (61) for a

recent study on the amides of the potato tuber.] The ninhydrin reaction (62) is now an important newer tool in this field.

Mothes (63) reviewed current theories of glutamine and asparagine synthesis in plants; Archibald (65) surveyed the chemical characteristics of glutamine and its role in plants and animals; Knight (66) reviewed the evidence that glutamine is an essential growth factor for bacteria. Barker (67) showed that glutamic acid is fermented by certain anaerobic bacteria liberating $NH_3$, $CO_2$, and $H_2$. Steward & Street discussed the roles of glutamine and asparagine in protein synthesis in the isolated tissue of potato tubers with particular reference to the part played by glutamine in "transamination." These recent summaries permit the reference to these substances to be confined to the points most relevant to the present discussion.

Differences between amide formation in tobacco and *Narcissus poeticus* have been referred to by Vickery *et al.* (5). Unlike narcissus, tobacco does not readily respond to ammonia by amide formation [c.f. (2)]. Narcissus plants which received nitrate to supplement their stored reserves grew better and formed more asparagine-amide nitrogen both in the light and dark than did nitrogen-starved, or even ammonia-fed, plants but the glutamine amide nitrogen content of the leaves did not respond appreciably to nitrate Vickery *et al.* re-emphasize their earlier view (26) that these two amides have different roles. Viets *et al.* (59) found that in corn plants asparagine was more responsive than glutamine to conditions which favor amide formation ($NH_3$ supply) or depletion. Street *et al.* (60) have found during the growth of isolated potato buds that the glutamine content is greater in the shoots than in the roots and in the shoots glutamine exceeds asparagine content. Both amides, but especially asparagine, increase in the shoots in response to ammonia supply but when the nitrogen-enriched plants were depleted they drew preferentially upon asparagine reserves. Again [c.f. (10)] asparagine appears as a reserve of soluble nitrogen in the potato plant and glutamine seems to be less concerned than asparagine with ammonia storage and to play a more intimate role in synthesis.

The properties of the glutaminase of the dog's kidney [Archibald (68)] and the demonstration of a similar enzyme in plant should stimulate work on plant amidases. If the hypothesis of

Schwab were experimentally examined this would naturally add to our knowledge of the respective roles of asparagine and glutamine [c.f.(61), pp. 180 *et seq.* ].

Mothes pointed out (63) that glutamine may precede asparagine accumulation in starved seedlings and in excised leaves and thereafter glutamine may disappear during protein degradation as asparagine accumulates. These changes are not entirely understood but they would be at least consistent if glutamine is more intimately associated with protein synthesis (accumulating in response to cessation of synthesis) and asparagine is more concerned with storage of the products of protein breakdown.[4]

Almost the whole of the amide fraction of beet tissue is glutamine and when the soluble-nitrogen of beet roots is artificially increased by ammonia the increase is almost quantitatively in the form of glutamine (69, 70). Neish & Hibbert (71) find that in beet discs under anaerobiosis glutamine decreases and asparagine increases. This recalls some cases in which glutamine occurs in leaves in the light but not in the dark [Vickery *et al.* on tobacco, and citations by Wood (2), p. 681]. Neish and Hibbert point out that the production of $\alpha$-ketoglutaric acid from citric acid requires oxidation but that asparagine could arise, not only from oxaloacetic acid, but also from fumaric acid derived from malic acid by processes which involve only the removal of water.

*Amino acids.*—The interrelationships of the free amino acids[5] in plants and their synthesis should be discussed in relation to parallel studies on animal cells.

In animals fed $N^{15}$ in the form of ammonia or amino acids the isotope concentration was greater in the dicarboxylic amino acid fraction than in any other amino acids except the one in which the isotope was supplied (72) and the concentration of $N^{15}$ was greater in glutamic acid than in aspartic. In tobacco plants supplied $N^{15}H_4Cl$ glutamic and aspartic were found to be richer in the isotope than the other amino acids: thus indicating their special role in the metabolism of both plants and animals (73).

[4] The catalytic role of glutamic acid in protein synthesis is suggested by Bonner's (64) observation that it acts as a cofactor in the alleged stimulus of wound healing by the hormone traumatin.

[5] A most promising technique for detection and analysis of amino acids, apparently not yet applied to plants, is the partition-chromatographic method of Martin *et al.* described in *Biochem. J.*, **38**, 224–32 (1944).

The mechanism of transamination [for review see Herbst (74)] focussed attention on glutamic and aspartic acid. These workers believed that transamination permits a general interconversion of amino acids; that the dicarboxylic amino acids or their keto acids play an essential role and that distinct glutamic and aspartic aminophorases are involved. Cohen (75) restricted effective transamination in animal tissues to the reactions of alanine, glutamic and aspartic acids with the corresponding keto acids and believed only one transaminating enzyme to be involved (76, 77). The only rapid reaction according to Cohen is that of glutamic and oxaloacetic acid to give aspartic and $\alpha$-ketoglutaric, which proceeds five times faster than the reaction of glutamic acid with pyruvic acid to give alanine and $\alpha$-ketoglutaric acids and very much faster than the reaction of aspartic with pyruvic. Lichstein & Cohen (78) demonstrated an active transaminating system in bacteria which catalyzed the reaction of glutamic with oxaloacetic acid: previous negative results (79) being ascribed to the attempt to measure transamination by the reverse reaction which is slow. Albaum & Cohen (80) showed that the interaction of glutamic acid with oxalacetic acid proceeded at a very rapid rate in oat seedlings and the reverse reaction at only one-third the rate. Activity in transamination preceded and outstripped protein synthesis and there appeared a close relationship between the two processes as well as to carbohydrate metabolism: the latter presumably being a source of nitrogen free recipients of amino groups.

Transamination is evidently widespread (81) but its role is more restricted (82) than the general and separate synthesis of the amino acid components of protein: the aromatic amino acids appear to be outside the normal scope of this process.

The evidence now is that transaminases are associated with pyridoxal phosphate as the prosthetic group (83 to 87) and that the process is to be visualized thus:

$$\text{glutamate} + \text{pyridoxal enzyme} \rightleftarrows \alpha\text{-ketoglutarate} + \text{pyridoxamine enzyme}$$
$$\text{oxaloacetate} + \text{pyridoxamine enzyme} \rightleftarrows \text{aspartate} + \text{pyridoxal enzyme}$$

Relations between the coenzymes of transamination and of amino acid decarboxylation (88 to 93) suggest other metabolic roles for substances in the vitamin $B_6$ group. Glutamic-aspartic transaminase from pig heart is sensitive to light [Schlenke & Fisher (84)],

though the light sensitivity of the enzyme from plants seems not to have been tested.

The keto acids are generally regarded as the immediate precursors of amino acids in plants [Chibnall (9)]. Either transamination or reductive amination, conceived as the converse of oxidative deamination, are the obvious methods of conversion. Transamination alone, however, seems too restricted in its application. Enzymic oxidative deamination of amino acids is of course familiar. The *l*(+)-glutamic dehydrogenase of plant and animal tissues, which oxidatively deaminates glutamic acid to $\alpha$-keto-glutaric acid, has been investigated by various workers (94 to 97). In liver and kidney Krebs (98) obtained evidence of an enzyme which could oxidize a wider range of amino acids and, as a *l*-amino acid oxidase, this has been further studied, obtained free from cell debris and electrophoretically homogeneous and shown to be a flavoprotein with riboflavin phosphate as the prosthetic group [(99,100) and references there cited]. Whilst transamination could account for the synthesis of the plant amino acids corresponding to $\alpha$-ketoglutaric, oxaloacetic and pyruvic acids, the formation of other amino acids may require mechanisms not yet positively identified as "reductive aminations" nor shown to be the converse of "oxidative deamination." Chibnall (9) demonstrated the quantitative conversion of the ammonium salt of $\alpha$-ketoglutarate to glutamine in perfused leaves of perennial rye grass: this, however, could be due either to the combined effect of transaminase and glutaminase in the leaf or to a reductive amination catalyzed by a *l*(+)-glutamic dehydrogenase or other enzyme.

That keto acids are the precursors of amino acids in plants, therefore, rests mainly on analogy with animal tissues. The keto acids known to occur in plants are pyruvic, oxaloacetic and $\alpha$-ketoglutaric acids. Recent references (101, 102) to the occurrence of pyruvic acid (in ***Allium***) are based largely on its color reactions. The positive identifications of oxaloacetic in plants are few and in the present state of knowledge indirect methods for the determination of $\beta$-keto acids are not unequivocal. The critical biochemical evidence for the occurrence of oxaloacetic acid in plants rests ultimately on Virtanen & Laine's (103) isolation of its oxime from legumes and their nodules and the conversion of the oxime (oximinosuccinic acid) to aspartic acid by reduction. Indirect evidence

that something like the Krebs carboxylic acid cycle is a widespread feature of plant metabolism is now impressive and all points to the occurrence in plants of its most probable intermediates. However, more knowledge is required of the plant keto acids and of the enzymes which mediate their interrelations with respiration (through carboxylation and decarboxylation) and with protein synthesis (through the corresponding nitrogen compounds). The recent survey by Vennesland & Felsher (104) of the plant decarboxylases is therefore pertinent.

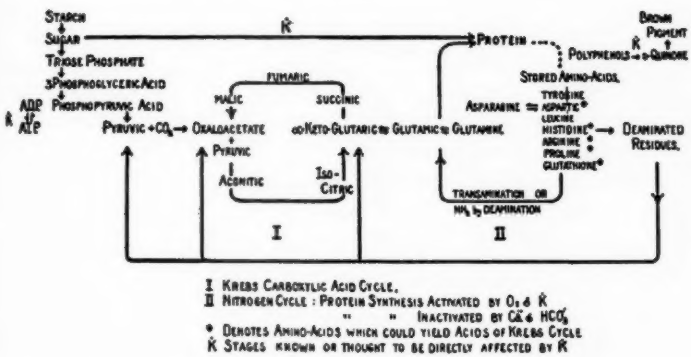


FIG. 1.—SPECULATIVE SCHEME OF METABOLISM IN POTATO DISCS AT 23°C.

Boswell (105) furnishes additional evidence that the polyphenol oxidase system of the potato tuber is responsible for the oxidation of amino acids; the amino acids being the hydrogen donors which reduce the *o*-quinones formed by the oxidase. This agrees with the point of contact between respiration and nitrogen metabolism in these cells advanced by Steward & Preston (10) and which is elaborated in Figure 1 of this review. Thus part at least of the respired carbon dioxide emerges from metabolic pathways directly connected with the nitrogen compounds.

While the dicarboxylic amino acids and alanine may well arise directly from the corresponding keto acids as products of glycolysis the synthesis of the other amino acids is much less obvious. There are still the two possibilities—the amino acids may arise

only by cleavage of proteins, synthesized by routes not necessitating separate amino acid synthesis, or each amino acid may have its own chain of separate synthesis enroute to protein. Neither possibility can yet be eliminated. It is impressive, however, that in liver and kidney several amino acids are converted to glutamic acid, viz., histidine, arginine, citrulline, proline, hydroxyproline and probably lysine (106 to 109). Glutamic acid is, however, not merely a product of amino acid catabolism; it is also a potential starting point for synthesis. Certain bacteria (*Staph. aureus*) use metabolic energy to accumulate glutamic acid from the environment but under the influence of penicillin this stops, respiration declines and the cells mature but do not divide (110).

A strain of *Neurospora* requires isoleucine and valine [Bonner *et al.* (111)]. The mixed keto acids corresponding to isoleucine and valine were ineffective in maintaining growth though either one of these keto acids with the other amino acid did promote growth. Tryptophane-requiring mutants of *Neurospora* have been described (112) of which one grows in presence of indole and the other in presence of indole and anthranilic acid though indole propionic, indolelactic and indolepyruvic acids are inactive (113). In this strain, the precursors of tryptophane are indole and serine. Tatum (114) showed that x-ray-induced mutants of *Escherichia coli* which require proline will grow without proline if they receive glutamic acid, though arginine, ornithine and hydroxyproline are ineffective sources. Thus, for these amino acids there is implied a much more subtle relationship between the amino acid formed and the source from which the organism derives carbon and nitrogen than the mere derivation of an amino acid from the corresponding keto acid.

The key position of glutamic acid in both amino acid synthesis and degradation is clearly evident: it is a substance to which breakdown proceeds and from which synthesis derives its nitrogen.

## Relation of Amino Acids and Amides to Respiration in Potato Tuber

It is possible to suggest, though not yet to prove, a mechanism by which glutamine metabolism may play a regulatory role in the tissue of the potato tuber. The system glutamine-glutamic acid now appears as a reactive intermediary in potato and to be es-

pecially fitted to transfer nitrogen groups to nitrogen free metabolites (61). Amongst the treatments which affect protein synthesis in rapidly aerated solutions of potassium salts there is a most suggestive contrast between the halides and phosphate on the one hand and bicarbonate-$H_2CO_3$ solutions at pH 7.0 on the other (10,11,12). The former treatment resulted in high respiration, oxidase activity, protein synthesis and salt accumulation: the latter depressed metabolism, suppressed the browning due to polyphenolase activity, stopped protein synthesis and salt accumulation (12).

In the light of carbon dioxide fixation, i.e., of the so-called Wood & Werkman reaction Steward (115) visualized the metabolic behavior of potato discs in terms of interlocking cycles (I &II) of metabolism (see Fig. 1). The Wood & Werkman reaction has not been shown for potato—this is a task for tracer-carbon—but it is not inherently improbable. The extremely rapidly aerated solutions which foster both protein synthesis and salt accumulation may, therefore, have limited the conversion of pyruvic acid to oxaloacetic acid and any other reactions which involve carbon dioxide addition. Equally aeration would promote decarboxylase reactions by removing any carbon dioxide so formed. Thus very effective aeration with $CO_2$-free air may now be visualized as tantamount to a block in the carboxylic acid cycle at the point of carbon dioxide addition.

Under these circumstances protein is synthesized in the potato discs and the nitrogen freed carbon framework of the stored amino acids forms a substrate for respiration—interchangeable apparently with the breakdown products of sugar now diverted to protein (13). The picture now is that at sufficiently low concentrations of carbon dioxide the carboxylic acid cycle operates, not alone by breaking down sugar to pyruvic acid which is fed into the cycle and metabolized to three moles of carbon dioxide per mole of pyruvic acid, but also by drawing into the system the nitrogen-free residues of glutamine and glutamic acid, i.e., $\alpha$-ketoglutaric acid. The freed amino groups of the system glutamine—glutamic acid are then in a form ready to be handed on to whatever receptive products derived from sugar may form protein. The system glutamine-glutamic acid could be replenished from the stored amino acids of the tissue.

In this way the otherwise obscure role of $KHCO_3$—$H_2CO_3$ at pH 7.0 becomes intelligible. The concept emerges of metabolic cycles which interlock, as it were, at the common point of glutamine-glutamic acid—$\alpha$-ketoglutaric acid and with carbon dioxide as a factor which controls their operation. Whether the low internal carbon dioxide concentration of green leaves in the light promotes protein synthesis in a similar manner is an interesting but unproven possibility: though depriving green leaves of external carbon dioxide in the light is said to have the converse effect and cause protein breakdown (116). Unexpected effects due to carbon dioxide (shift toward alkalinity of the sap) are known (117,118) and some of these involve hydrolysis of glutamine to glutamic acid. Apparently in the beet carbon dioxide fosters amide hydrolysis: in the potato (12) it depresses protein metabolism, causes amide (glutamine) to accumulate and conserves the storage amino acids [(12), p. 495].

The connection between respiration and nitrogen metabolism may take a different form in other systems (mature isolated leaves) characterized by protein breakdown. In the work of Gregory (8), Yemm (119), Vickery (120), Wood (2,57), and their respective collaborators, the relation between the destructive metabolism of amino acids and respiration is apparent. The general feature is that deaminated residues, drawn into the metabolic cycles from which carbon dioxide emerges, stimulate respiration. In the potato tuber the existing amino acid concentration does not play the determining role in respiration, but it is through the amino acid metabolized, furnishing at the same time nitrogen for protein and a carbon-residue for the respiration cycle that this respiratory effect is created.

## Soluble Nitrogen Fractions Other Than Amino and Amide-Nitrogen

The nonprotein organic of plants, which is not included in the amide and amino nitrogen, is often termed "other" or "residual" nitrogen and may be large (58,59). The "other" nitrogen will include polypeptide and peptide nitrogen; the nonamino nitrogen of arginine, histidine, proline, and tryptophane; purine nitrogen and the nitrogen of the simpler natural bases, alkaloids, and glycosides. In addition to the familiar inorganic forms of nitrogen

hydroxylamine, free and combined as oxime, also occurs in small amounts in plants. Attention is here restricted to those forms of residual nitrogen which have some special interest at the present.

*Nitrogenous bases.*—The simpler natural bases, the metabolism and chemistry of which were reviewed by McKee (121) and Guggenheim (122) respectively, have special interest because they are ubiquitous in cells. Choline as a constituent of lecithin has long been regarded as universal—Klein & Zeller (123) found free choline in all but three (which were lichens) of one hundred genera examined. The vitamin-like activity of choline in the animal may follow from its role as a methyl donor [du Vigneaud (124); Bach (125)] and some evidence has been obtained for the early view of Trier (1912) that its precursor in the animal body is aminoethanol.

Noncholine producing strains of the ascomycete ***Neurospora*** *crassa* [Horowitz & Beadle (126)] enabled Jukes *et al.* (127) to show that choline is formed by stepwise methylation of aminoethanol. Horowitz (128) showed that of two cholineless strains one (34486) fails to make monomethylaminoethanol from aminoethanol, but can complete the methylation to choline if the intermediate is supplied. The other strain of cholineless *Neurospora* (47904) cannot use monomethylaminoethanol as an intermediate in choline synthesis. The ability of higher plants to use aminoethanol for choline synthesis has not yet been demonstrated.

The role of glycine-betaine, detected in higher plants by Stanek in 1906 (129) has long been obscure. Barrenscheen & von Vályi-Nagy (130) claim to have demonstrated the methylation of glycine to betaine in wheat germ in presence of methionine—the sulphur of which was oxidized. Betaine may act as a methylater in animals and there is evidence (131) that demethylation of betaine and methylation of aminoethanol may be coupled. The biological function of choline oxidase (132, 133) which converts choline to betaine aldehyde and probably even to glycine-betaine is not clear. Klein & Linser (134, 135, 136) investigated the formation of betaines following the injection of plants with various nitrogen compounds and thence formulated a scheme of reactions showing the interrelationships of glutamic acid, proline, ornithine, nicotinic acid and the betaines trigonelline and stachydrine.

The relative neglect by plant physiologists of the simpler natural bases is due to the small amounts in cells and to the lack of

convenient methods of estimation. Improved methods, recently applied to the investigation of animal metabolism, are now applicable to plants. The reineckate precipitation method for choline (137, 138, 139) is only selective for quarternary bases at pH 12 to 13. At this reaction the precipitates are somewhat intractable and decompose: Street *et al.* (140) have applied an earlier method of Lintzel & Monasterio (141) to plant extracts subjected to a preliminary reineckate purification. Microbiological methods using noncholine producing strains of *Neurospora* (142, 143) have been described. Artom (144) has described a method for aminoethanol. The determination of betaines (145, 146) still remains difficult.

*Purines.*—Though the purines are concerned together with the pyrimidines in the formation of nucleic acids, discussion of these cyclic compounds would carry this review too far into a special field. Attention is however directed to some recent methods of analysis for purines and related compounds; for purines (147, 148); for allantoin (149, 150); and for nucleic acid (151 to 154).

*Alkaloids and glycosides.*—Part of the soluble nitrogen may consist of alkaloids or glycosides. The synthesis and role of nicotine in the tobacco plant has been reviewed by Dawson (155) and papers on the metabolism of alkaloids in *Atropa belladonna* and other solanaceous plants have been published by James (156) and Cromwell (157, 158). Nicotine (155) like the alkaloids of *Atropa* (157) and *Datura* (159) seems to be synthesized in the root though limited synthesis may also occur in detached leaves of belladonna (156) and independent anabasine synthesis in *Nicotiana* can occur in both root and shoot. James[6] (156) stresses that alkaloids and protein synthesis proceed concomitantly in the growing regions though detached leaves, incapable of protein synthesis, may profit temporarily by the amino acid from protein breakdown to synthesize some alkaloids.

Views on the path and intermediates of alkaloid synthesis can be found in the papers of Cromwell (158), James (156), and Dawson (155). That tetraploid *Nicotianas* have a greater relative (i.e., per cent) nicotine content than diploids [Noguti *et al.* (161, 162)] and that polyploid *Daturas* have increased alkaloid content (160) is

[6] Also see James, W. O., *Nature*, **159**, 196–97 (1947).

another of the many recent examples of the common ground between cytogenetics and biochemistry.

The estimation of solanine, a glycoside of potato plants (163, 164), and its distribution during development (22, 140, 164, 165) have been investigated. The metabolic role of this substance, other than as a widely fluctuating storage product (which accounts for only a minute fraction of the "other nitrogen"), is still obscure.

*Nitrite-, hydroxylamine-, and oxime-nitrogen.*—Soluble nitrogen in plants may exist in relatively small amounts as free hydroxylamine and as oxime and although only present in small amounts the metabolic interest of these substances may be disproportionately large in comparison with the more stable soluble storage products.

Virtanen's contributions to the mechanism of nitrogen fixation [reviewed by Wilson (166)] continue (167 to 170) to focus attention upon the role of hydroxylamine and oximes in nitrogen fixation by bacteria and to suggest their more general role in nitrogen metabolism. Wilson remarked [(166), p. 176] that although most writers reserve for ammonia the role of the primary inorganic substance which enters the cycle of protein metabolism, more critical investigations should be carried out to test the claims for hydroxylamine. There are many reasons for an experimental reexamination of the ammonia hypothesis in higher plants and of its alternative, the oxime-hydroxylamine hypothesis, but ability to satisfy this need is limited by the routine methods available for the analysis of the alternative forms of nitrogen.

One of us[7] encountered unexpected difficulty in the application of the aniline method of Ostern (171) & Edson (172) to the analysis of keto acids in alkaline plant extracts prepared after the method of Virtanen. For reasons which are here immaterial the aniline reaction was performed on the acidified, evacuated solution in a Van Slyke constant volume apparatus and on microanalysis the gas evolved proved to be largely inert (60 per cent not absorbed by alkali, alkaline pyrogallol or cuprous chloride). The general parallelism between this behavior and the keto acid content of the extracts as shown by the Friedeman & Haugen (173) method led to

[7] FCS working with R. A. Whitmore—unpublished work done in Botany Dept., University of Chicago, Chicago, Ill.

the view that the gas (nitrogen) originated in the acidified extracts by spontaneous oxidation of substances (probably oximes) to nitrite which then reacted with amino-groups

The basis of determinations of nitrite in plants is the Griess reaction (sulphanilic acid and naphthylamine): the basis of the determination of hydroxylamine is the Blom reaction (oxidation of hydroxylamine to nitrous acid by iodine in glacial acetic acid). Lemoigne *et al.* (174) prescribed the application of the Griess and Blom reactions to lead cleared solutions in order to distinguish qualitatively between free nitrite, free and combined hydroxylamine (oxime), the latter being hydrolysable to hydroxylamine and then oxidized to nitrous acid. Free nitrite is removed by adding urea in acid solution, and the Blom reaction is applied to the nitrite free solution both with and without acid hydrolysis. Lemoigne *et al.* stipulate that the color with sulphanilic acid should be developed in solutions the acidity of which is regulated with sodium acetate. For qualitative work this is probably enough but to make the methods quantitative, more careful attention is necessary to the pH of the reaction mixture and, if this is controlled between narrow limits (around pH 2.8), the color can be read ($\lambda = 535$) in a Coleman or other suitable spectrophotometer. The conditions for optimum hydrolysis of oxime with minimal loss of hydroxylamine and for the Blom reaction also need to be standardized in quantitative work. Rider & Mellon (175) have described the quantitative use of the Griess reaction for nitrites.

With plant extracts all these methods encounter further difficulty. Though the oxime fraction can be stabilized in cold alkaline extracts the Griess and Blom reactions require colorless protein-free solutions which are only obtained satisfactorily by clearing the alkaline extracts with neutral lead acetate. Even after this cumbersome procedure leaf extracts of legumes (bean, alfalfa) commonly contain not only nitrite but also hydroxylamine both free and combined. Table I is suggestive, though in view of the analytical difficulties only suggestive, of an interrelationship between these nitrogen compounds and the keto acids of the same extracts deproteinized with trichloracetic acid (here called free keto acid) or deproteinized and hydrolyzed with hot mineral (sulphuric) acid (free and combined keto acid).

This leads to two other topics, the binding power of tissue proteins for certain metabolites [cf. (176)], and the losses of elementary nitrogen by plants during metabolism, and owing to space restrictions, these problems are stated in skeletal form.

*Binding power of tissue proteins for metabolites.*—When groups can only be detected after the removal of the protein from tissue

TABLE I

CONTENT OF $NO_2^-$, $NH_2OH$ (FREE AND COMBINED) AND OF KETO ACIDS (FREE AND COMBINED) IN CERTAIN PLANTS MATERIALS. ALL QUANTITIES IN MICRO-MOLS. PER GM. FRESH WT. OF TISSUE

| *Sample* | Free $NO_2^-$ | Free $NH_2OH$ | Combined $NH_2OH$ | $NO_2^-$& total $NH_2OH$ | Keto acid | Total keto acid after hot acid hydrolysis |
|---|---|---|---|---|---|---|
| Field grown alfalfa leaves stored in dry ice prior to extraction | 0.22 | 0.035 | 0.371 | 0.63 | 3.66 | 6.03 |
| Opposite leaves of Phaseolus greenhouse grown: extracted in sand and cold alkali | 0.21 | 0.100 | 0.191 | 0.50 | 1.54 | 2.83 |
| Ditto but young 2nd trifoliate leaves | 0.37 | 0.147 | 0.165 | 0.68 | 2.10 | 5.10 |

extracts there is a real dilemma to know how far a given metabolite (such as keto acid) existed free in the extract before deproteinizing or in the cell and to what extent it was previously bound to protein.

The keto acids liberated by cold trichloracetic acid are a large part of those determined after warm mineral acid (Table I) which may hydrolyse protein. How far this represents displacement of keto acids from protein combinations and how much it is due to their formation during protein hydrolysis is a moot point.

*Losses of elementary nitrogen.*—Pearsall & Billimoria (177) redirected attention to this phenomenon in their work with daffodil (*Narcissus pseudo-narcissus*) leaves: Vickery (5) detected losses of nitrogen in developing leaves of *Narcissus poeticus* which he attributed to the oxidation to nitrite of ammonia from deamination of amino acids. The route hydroxylamine plus keto acid→oxime→amino acid [which is a well known method of preparing amino acids *in vitro* (178)] may thus be more general in flowering plants than has been supposed for, if catalyzed *in vivo*,[8] the hydrolysis and oxidation of oximes could also lead to nitrite and thence to losses of nitrogen. An outstanding need is for reliable routine methods to determine hydroxylamine and oxime-nitrogen and also of the plant keto acids with which they are so intimately connected. A definitive contribution to this field is that by Virtanen *et al.* (167).

*Nitrate and ammonium in nutrition.*—The detection of nitrite in plants during active nitrate assimilation [Eggleton (179)] supports the classical views that nitrate reduction proceeds via nitrate-nitrite-ammonia or by the alternative steps [Chibnall (9)] nitrate-nitrite-hyponitrous acid-hydroxylamine-ammonia. In either event ammonia is assigned the central role in the primary synthesis of nitrogen compounds. Nitrate, however, must still be regarded as the most important source of nitrogen for cultivated plants, though with deficient nitrification, due to poor soil aeration etc., ammonium salts occur and are utilized [Vickery (6)]. Usually in culture experiments nitrate proves to be a more efficient source of nitrogen than ammonium. Vickery *et al.* (5) found this also with *Narcissus poeticus*. Shive (180) and Gilbert & Shive (181) showed that the response of ammonium salt absorption to aeration was greater than that of nitrate and later showed (182) that at a given low oxygen tension roots supplied nitrate respired at a higher rate than those receiving ammonium. In *Nicotiania* the absorption and metabolism of nitrate stimulates the formation of organic acids from sugars [Vladimirov (183)]. Leonard & Pinckard (184), however, did not find nitrates more favorable than ammonium salts to cot-

[8] Virtanen *et al.* (168) regard *l*-aspartic acid as the "grundamino-saure" of legumes because its oxime forms at pH 5.3 three times as fast as that of $\alpha$-keto glutaric acid. Hydrolysis and oxidation of oximes *in vitro* is a maximum at pH 2 to 3.

ton roots at low oxygen tension. It is characteristic of the roots of plants receiving nitrate, however, that they are relatively poor in amide and amino nitrogen and relatively rich in "other nitrogen." This has led to the suggestion that the actual course of synthesis is different according as the source is nitrate or ammonia [cf. Sideris & Young (185)] as would be the case if nitrate were converted to some rather complex organic form without first being reduced to free ammonia.

*The role of light.*—The root (and other growing regions) and the green leaf exposed to light are the main seats of protein synthesis. These two centers are so different as to presuppose different metabolic pathways to protein. From Baudisch (186) to Baly (187) the photochemical production of organic nitrogen compounds between activated formaldehyde and nitrites has been a speculative explanation of synthesis in the leaf. Later (188) Burström submitted the problem of nitrate assimilation to comprehensive review. In nitrogen autotrophic, carbon heterotrophic organs (roots and especially root tips) or organisms (fungi) the assimilation of nitrate is held to be linked, energetically, with the aerobic breakdown of carbohydrates and it ceases at anaerobiosis. Nitrate reduction and assimilation in these systems is catalyzed by heavy metals (manganese in wheat roots; molybdenum in *Aspergillus*). By contrast, the green leaf (carbon autotrophic) reduces nitrate in the light by processes which are specifically linked with reduction of carbon dioxide. In this process the "source of energy must be light and not respiration and the simultaneous assimilation of carbon-dioxide delivers the carbon compounds of which protein is built up." Burström recognizes that the assimilation of ammonium proceeds in the dark at the expense of stored sugars and that it is specifically the reduction of nitrate in the light that is concerned in some way with the photochemical reduction of carbon dioxide. Carbon heterotrophic organs or organisms produce carbon dioxide which is unaccompanied by an equivalent oxygen absorption and this "extra carbon-dioxide" is in effect due to the oxidizing ability of the nitrate which is reduced [cf. Gilbert & Shive (182)].

From the relative stability of the soluble nitrogen against side reactions (loss of nitrogen by interaction with nitrous acid) Burström postulates that the principle processes of nitrogen assimila-

tion, whether light activated, or not, occur while the radicals are bound to some protoplasmic constituent. Though bound these radicals may remain in reversible equilibrium with the ions present in the cytoplasmic fluids.

Burström does not specifically identify the "protoplasmic constituent" to which intermediates of nitrate reduction are attached. Burström's "intermediates at the oxidation level of hydroxylamine" may well be combined with keto acid and probably also bound to protein. In fact the suggestion that these lesser known nitrogen and keto acid fractions may fulfill their functions as intermediates of metabolism while in actual combination with protein has much to commend it.

*Protein metabolism and related processes.*—The view that synthesis proceeds step by step by condensation of amino acids leads naturally to formulation of the protein level in terms of equilibria and of reactions which are controlled by the concentration of reactants.

Wood *et al.* (189, 190) suggest that the direct relations between proteins and amino acids in starving leaves may be displaced by the preferential oxidation of cystine and Wood *et al.* (191) have traced the concentrations of different amino acids which result from protein breakdown in starving monocotyledon leaves. The salient point is, however, that these mature isolated monocotyledon leaves do undergo such rapid protein breakdown accompanied, even by the second day, by some obviously morbid changes. One must presuppose that synthesis is the reverse of proteolysis, i.e., accept the essential point at issue, to apply findings from these systems to those in which synthesis occurs. However, enzymic re-synthesis of polypeptides[9] from protein hydrolysates is still too far removed from the behavior of cells to be a very strong argument in favor of the amino acid hypothesis of synthesis *in vivo*.

The alternative view of protein synthesis regards the step by step condensation of amino acids *in vivo* as inherently improbable and the evidence for the converging synthesis of the amino acids required to make up plant protein as meagre. Protein synthesis and proteolysis are regarded as distinct processes—not merely aspects of a reversible system. These concepts seem necessary to explain the metabolic behavior of barley leaves [Gregory & Sen

[9] See Bergman, M., and Fruton, J. S., *Advances in Enzymol.*, 1, 63–98 (1941).

(8)] and of potato discs in aerated solutions [Steward *et al.* (10 to 14)].

The crucial problem of protein synthesis surrounds the mechanism of energy coupling. Basically the picture is as follows: When Harden & Young first recognized the role of phosphorylation in the breakdown of sugar it was conceived that the phosphate rendered the sugar molecule compatible with the enzymes of fermentation. The modern view is that the primary ester type of phosphate linkage may be changed during metabolism into an "energy-rich" phosphate linkage, i.e., a linkage in which phosphate is attached to nitrogen (as in phosphocreatine) to another phosphate (as in a pyrophosphate) or to a carboxyl group (as in an acylphosphate). In the metabolism of muscle the energy of metabolism is used to create the "energy-rich" phosphate linkage (creatine phosphate) which is then transferred at the high energy level through the medium of adenosinediphosphate to triphosphate from which the energy is liberated in a form which can be absorbed by the muscle protein in the actual process of contraction, or more precisely, of relaxation. Admittedly protein synthesis in a meristematic plant cell is far from the mechanical work of a muscle fiber but the generality of the principles enunciated by Kalckar (192) and Lipmann (193) is now so widely recognized that it would be surprising if the transfer of energy in synthesis were not effected through some energy-rich group containing bonds which may be generated during metabolism from energy-poor compounds (esters or inorganic phosphates).

Bergmann & Fruton [(194), pp. 412–17] considered the possibility that part of the energy liberated in hydrolysis of certain peptide bonds (order of 3,000 cals.) may be provided in synthesis by coupling synthesis with phosphorylation and they pointed to the effect of phosphorylation on the stability toward proteolytic enzymes of a dipeptide (seryl-glutamic acid). Proof that phosphorylation is directly involved in primary protein synthesis in plants still waits upon positive isolation and identification of the phosphorylated nitrogen compounds.

There is, however, no lack of evidence to connect the metabolism of phosphorus and of proteins in plants [c.f. (3, 4)]. Both the respiration and the protein synthesis of potato discs in aerated solutions were increased by phosphate concentration at pH 7.0 in ways which implied a specific response to phosphate (12) which

Steward & Preston postulated was exerted through the nitrogen compounds rather than solely through phosphorylated sugars.[10]

An explanation of the energy linkage in the protein synthesis of potato discs should also explain ion accumulation with which it may be linked in growing cells. One course of speculation is as follows: A phosphorylated energy-rich nitrogen compound may be formed at the outermost plasmatic surface in contact with free oxygen using the energy of metabolism (respiration) in its formation. Such a phosphorylated nitrogen compound might possess amphoteric properties and thus be able to escort both anions and cations across the protoplasms. At the seat of protein synthesis the energy bound in the phosphate linkage would be released in a form in which it can be utilized by the protein forming surface in binding the nitrogen groups which are also furnished in a form compatible with synthesis—meanwhile the associated ions freed from the complex would be released in free solution into the vacuole. Such views are entirely speculative but they do suggest the lines along which explanation may be sought of the undoubted connection in plants between a component of the aerobic respiration as a source of energy, protein synthesis from stored reserves of amino acid, and salt accumulation in the vacuole.

[10] See also the nutritional studies of RICHARDS, *Ann. Botany* [*New Series*] **2**, 491–534 (1938).

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# MINERAL NUTRITION OF PLANTS[1]

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This review is restricted to a few specific questions, namely, the mechanism of absorption and transport of ions in cells and tissues, the mechanism of active absorption and transport of water (which is believed to be intimately correlated with the movement of salts), the relation between the concentration of salt nutrients in certain tissues and growth (foliar diagnosis, etc.), and the biochemical effects of certain nutrient elements.

## Exchange of Ions between Cells and the Surrounding Medium

The problem of absorption of mineral salts in single cells and their transport within multicellular organisms still occupies a central place. The salts, as a rule, are absorbed against an osmotic gradient, which thus postulates that work must be performed by the cell.

Even if the absorption of salts in living cells is a deeper problem than that of common permeability, the entrance of ions through the cell surface always emphasizes permeability problems. A membrane of pure oil would not let ions through. Even the recent ideas of a lipoid film superposed by a thin layer of protein (1) leave the process of ion absorption unexplained. The surface of the protoplasm is certainly complicated, something of a mosaic pattern, especially in young growing cells which absorb salts actively. Since the mineral salts are absorbed as ions, not as molecules or ion pairs, the assumption of a certain ion activity in the cell surface itself becomes plausible. According to this theory the primary phase of salt absorption is an ion exchange at basic and acid groups in the protoplasmic membrane. This is supported by experimental research on the relation between the surface potential of the roots and the ions in the medium (2,3), and on the exudation of organic compounds from the roots (4). The normally negative charge of the root surface is due to the dominance of strongly acid groups, possibly nucleotides.

[1] This review covers the period from October, 1946 to October, 1947.

The primary phase of the ion absorption being an exchange between the hydrogen ions of the acid groups and the cations of the medium, and between the hydroxyl ions of the basic groups and the anions of the medium, the surface layer of the protoplasm will hold a quantity of "adsorbed" ions of more constant concentration than that of the medium. The potential difference between the cell and the medium is chiefly of a Donnan character, and diffusion potentials, though theoretically not excluded (7), are of minor importance. All ions participate, of course, in the electrochemical equilibrium and the assumption of an exceptional influence of the hydrogen ions is an absurdity (8). The hydrogen ions, however, have a high exchange power in comparison to a number of metallic cations, a fact which controls the intensity of exchange. At sufficiently high concentration of neutral salts in the medium the cell surface is nevertheless discharged, and the same is, of course, the case if the $C_H$ of the medium is raised to the same height as the $C_H$ of the cell surface (in the case of roots of cereals at pH 3 to 4). A reversal of the charge from negative to positive was observed on *Nitella* after treatment with KOH (9). The idea of the amphoteric character of the protoplasmic membrane is not in opposition to the theories of permeability of nonelectrolytes by the means of a lipoid-protein film, because the nondissociated ground substance of the membrane probably does not interfere with ion absorption. Chemical linkage of the basic and the acid parts of a salt to special substances in the protoplasmic membrane was proposed earlier (10), but not in such a form that it could be combined with the experimental facts on active salt absorption by roots. Experimental work in surface chemistry, including the reactions of monomolecular films, will contribute to a more accurate picture of the plasma membrane.

Studies in the absorption of salts in slices of storage tissue have also resulted in the assumption of a primary phase of a nonmetabolic character (11). The ions, which are adsorbed in the surface, are no doubt also able to enter the protoplasm spontaneously, provided that adsorption potentials are exhibited throughout. The protoplasm as a whole can be regarded as an amphoteric colloid with dominating acid dissociation (the isoelectric point is normally on the acid side) and it adsorbs consequently cations, which may be transferred from the surface by "turning over" of ionized large molecules, along "ion tracks," e.g., ionized side-chains of

proteins, or by protoplasmic streaming, in any case much faster than by diffusion. The rapid transport of cations through the protoplasm has been demonstrated with radioactive isotopes (16). It is a well-known fact that the balancing effect of an assortment of cations, preferably $K^+$ and $Ca^{++}$, on negative valence points of the protoplasm is one of the necessary conditions of the proper structure and consistency of the protoplasm.

The exchange of cations between the medium and the bulk of the protoplasm proceeds also in nitrogen and is consequently independent of the oxygen respiration (5). It is nonpolar and proceeds from the medium to the cell or from the cell to the medium according to the prevailing concentration gradients. Exchange of cations was studied on *Chlorella pyrenoidosa*, the protoplasm of which possibly contains different organic compounds of very low mobility, with specific activity for sodium, potassium, calcium, and magnesium (12). Cationic exchange was also studied in *Chara* using radioactive isotopes (34). The remarkable fact that a corresponding nonpolar exchange of anions was seldom observed is probably due to the anionic character of the protoplasm as a whole, in consequence of which the membrane equilibrium is strongly displaced to the disadvantage of the free moving anions. Thus, the exchange of tagged $PO_4$-ions proceeds with difficulty (132). The negative surface potential of roots even favors the exudation of large anions, e.g., nucleotides (4). Finally, the mineral anions are metabolized more rapidly than the cations.

With regard to nonpolar exchange of ions, diffusion probably plays a minor role, and exchange is limited by special conditions. In distilled water few ions are present and the loss from the cell is consequently small unless the water contains appreciable quantities of carbon dioxide ($H^+$ and $HCO_3^-$ ions as exchange material). As a consequence "aeration" of the medium has a large influence on the loss of ions. All these experiments must of course be performed in the absence of oxygen, viz., at inhibited salt respiration. These facts may aid in explaining seemingly controversial results on the reversibility of salt absorption (35).

The surface of the protoplasm is apparently not solid, but a film of oriented molecules showing many characteristics of the solid state. The behavior of the surface of roots shows several analogies with collodion membranes. These are more or less impermeable to anions, owing to the presence of large molecular

groups, mostly impurities, which permit exchange of cations (13,14). Models of interfaces between different oils to which dissociable dyes were added show potentials created by one-sided accumulation at the interface (15). Still better models are collodion membranes prepared with phospholipids (17). These membranes owe their potential to the ionization of the fixed molecules of lecithin or cephalin. Nonionized acids and bases permeate easily through these membranes, ions more slowly.

Observations on bacteria support the assumption of electronegative groups in the surface of the cell. Bacteria show exchange of cations (18), and adsorption of cations is characteristic of gram negative bacteria (19). The dye reactions are ascribed to the presence of a ribonucleic acid cytoskeleton and its state of oxydoreduction. The ribonucleic acid is believed to be combined with magnesium and protein (21). The plasma membrane of certain bacteria gives a distinct Feulgen reaction (133). The existence of combined groups on the surface of bacteria has been mentioned to explain the action of certain disinfecting agents (19) through absorbable cations. In the case of interchange of sulfonamide and *p*-aminobenzoic acid on the surface of *E. coli* the active groups are believed to be basic (22). Protoplasmic membranes capable of cation exchange, but impermeable to anions, have been described in *Amoeba* and *Rhizoclonium* (23). The protoplasm of *Chara* is also permeable to cations (24). These membranes, similar to the charged membranes of higher organisms, are freely permeable to undissociated salts. The theory of an ion activity on the surface of the protoplasm, developed on the basis of researches on the mineral nutrition of roots, thus seems to have a wide scope. The exchange of ions in the surface of roots was shown to obey approximately the law of mass action (3) and the linkage of ions to large protein molecules also obeys this law (25). All attempts to explain observed phenomena, e.g., potential differences, absorption and exchange of ions, from physicochemical principles, such as membrane equilibria, diffusion stages, mass action, etc., are as yet approximations, because the activities, hydration, etc., of the reacting ions and the molecules are seldom sufficiently known, and because true equilibria never occur. Recent experiments point to a high complexity of many membranes. From observations of the strong correlation between activity of hydrolyzing enzymes of yeast cells and the pH of the medium it was concluded that these

enzymes (lactase, invertase, etc.) are localized on the surface of the cell. Similar results were yielded with respect to the invertase activity of roots (27). Here the pH of the plasma membrane itself, which is regulated by cation exchange, determines the enzyme activity [the local pH of large molecules is sometimes called $pH_S$, see (28)]. A rather complicated structure of the membrane is deduced from the almost total impermeability to sodium observed in many cases, whereas potassium generally rapidly penetrates the cell surface. Different chemical potentials have been considered in this respect (29,30), but another possibility is the blocking of active components of the membrane by ions of special size and electronic configuration. Curious cases of selectivity have been observed in the tentacles of *Drosera capensis* (31). Selectivity can also be caused by specificity of the active metabolic processes involved in salt absorption. Processes which regulate the accumulation of sugar are apparently localized at the surface of the roots (32).

Observations on roots and regenerating storage tissue have shown that the active uptake of ions is preferentially connected with growing cells. This may be due to an intensification of the aerobic respiration system responsible for the accumulation energy, although the capacity of exchange of the protoplasmic surface may have an influence. The fact that acid nucleotides are preferentially exuded by the still growing parts of the roots may mean that ageing of the cell implies a steady de-ionization of the surface, viz., a disappearance of the active groups controlling ion exchange. There may also be a correlation between the strong ion activity of the protoplasmic membrane and the processes of wall formation. Owing to the abundance of strongly acid substances in the interface between protoplasm and wall this process is buffered somewhat against variation of the salt content of the medium and of the pH. The buffers may be adsorbed Ca-ions or Ca-pectinates (33).

## Active Accumulation of Ions

As stated above, a certain "passive" ion exchange occurs between the protoplasm and the medium. This exchange is not directly comparable with the simple diffusion permeability of nonelectrolytes, and it is presumed that the transference of ions is localized to special areas of the membrane.

A true accumulation of a neutral salt obviously cannot result from ion exchange only. Cations are certainly spontaneously accumulated owing to the prevailing anionic character of the protoplasm, which counteracts the accumulation of movable anions. In the absence of a special mechanism for accumulation, the storage of cations in a cell would vary greatly owing to variations in the concentration of the medium and the general balance of ions (also including $HCO_3^-$). The concentration of cations and of the anions of mineral acids is in reality fairly independent of variation in the medium. This suggests a "steady state" between passive "leakage" and active uptake of ions, considered by the writer (36) and which has gained recognition. The discovery of a special respiration system sensitive to cyanide, controlling the accumulation process (37) has been corroborated (38,39,40). The distinction of a second respiration system, the "ground respiration," insensitive to cyanide, and independent of salt uptake is also accepted.

The causality between salt uptake and respiration has been affirmed by several investigators, but there is disagreement as to its nature. On one side the assumption was advocated that the aerobic metabolism (or a part of it, e.g., the protein synthesis) constitutes a general unspecific condition for salt accumulation (41), whereas other investigators (5, 36, 37) pointed out a mass relation between the quantity of absorbed salts and the intensity of the respiration. The mass relation was originally demonstrated by use of growing roots, the accumulation power of which is well developed and far exceeds the requirements of the cells. Since similar results are available in the case of slices of storage organs (38, 39, 40), the earlier polemics (42) against the theory of ground respiration and salt respiration have lost authority. Respiration mechanisms sensitive to cyanide also occur in leaves of barley (43).

The further development of the theory is due to experiments concerning the varying relations in the absorption of anions and cations, which are caused by the nature of the salt and the storage of salts in the organ before the start of the experiments. Respiration is far more intimately related to the absorption of anions than to the absorption of cations, as a consequence of which the term "anion respiration" has replaced "salt respiration." The potentials are created through the active absorption of anions, which "passively" attract the cations (43). That anions influence the absorp-

tion of basic substances has been frequently demonstrated, e.g. (44), and the fundamental assumption of different points of absorption for anions and cations is in agreement with the chemical views of absorption of salts in amphoteric colloids.

Negative potentials in the protoplasm are, of course, not exclusively created by anion respiration. It has been observed, for example, that potassium enters the cell almost independently of the oxygen content of the medium (45). Such cases usually refer to the production of organic acids in the cells. Numerous investigations bear evidence of the high buffer capacity of the root cells against changes of pH. In addition to moderate buffering around pH 6.4 owing to carbon dioxide, the root cells are endowed with an effective metabolic buffering system, in which the production or desmolysis of malic acid predominates (46). The content of malic acid in the roots of wheat is always almost equivalent to the excess of mineral cations over mineral anions present in the sap, regardless of the balance between the ions during the absorption. Nitrate and ammonium salts do not form exceptions to this rule. This amplification of earlier investigations (47) disproves suppositions (48) that anion respiration might be related to the disappearance of organic acids. It has been experimentally shown (32) that the consumption of glucose rises, if the roots are held aerobically in a solution of potassium chloride, whereas the addition of potassium chloride to a glucose solution in nitrogen did not increase the consumption of carbohydrate. In the last case, according to earlier experiments, only traces of $Cl^-$ are absorbed, whereas in oxygen a measurable absorption of chloride occurs. The anion respiration thus implies a desmolysis of glucose, not a combustion of organic acids. This is substantiated by the constancy of the ratio, anion respiration to anion absorption, for nitrate absorption from calcium nitrate, or from potassium nitrate. In the former, cation absorption is negligible, whereas in the latter it is appreciable [(36, Table X].

The prevailing anionic character of the protoplasm creates a "permanent" absorption potential for cations. Such are the *a priori* conditions for the accumulation of cations, but the accumulation of a free neutral salt postulates a comparable internal concentration of free anions which postulates a special mechanism. In a cationic protoplasm the cations would require an extra supply of

accumulation energy. Such cases have not been hitherto satisfactorily demonstrated. The anion respiration is apparently the usual process of salt accumulation in plant tissues.

Starting from the fact that the oxidation mechanism of a cytochrome-cytochrome oxidase system implies a change of valence of iron atoms the writer (5, 6, 43) pictured the transport of anions as a corollary to the transference of electrons through an enzyme system. The change of valence of the iron must be balanced by a periodically occurring attraction and repulsion of anions. Absence of dissociated salts acts as a brake on the mechanism. A series of active iron atoms separating two media of different redox potentials will transport anions from the medium of higher to one of lower oxidation power. In cells or tissues, ions will, in general, be transported in direction from the outside to the interior. This hypothesis explains the transport of all anions, and that the presence of free anions is a condition for the functioning of the respiration mechanism. It also explains why no stoichiometric relations exist between the transported anions and the liberated carbon dioxide. However, a semiquantitative mass relation exists, the fast moving ions being favored during the transport. If the transport of anions is an accessory phenomenon to an enzymatic desmolysis of glucose, this of course leaves room for seemingly causal relations between salt accumulation and synthetic processes for which respiration furnishes the energy. The proposed hypothesis may serve as a basis for continued experimental inquiry. It is deplorable that the theory of anion respiration and the extensive experimental work, by which it is supported, was dismissed without discussion in a recent work on cell and tissue physiology [(49), p. 594].

## Salt Transport and Sap Movement

Recent investigations on the mechanism of bleeding (5, 50) convey the conclusion that the anion respiration is not restricted to a pumping device at the surface of the root, but works in the whole cell layer between the epidermis and the central vessels, thus maintaining a continuous inflow of salts. An oxygen gradient, demanded by the above hypothesis, does exist in most roots. From the respiration values of wheat roots the diffusion coefficient of oxygen was calculated at approximately $10^{-6}$ cm$^2$ sec$^{-1}$ (51) and the concentration of oxygen in the center of the root is believed to sink

to zero for intensive respiration. Analysis of the intercellular air [(50), p. 26] gave higher values, however. Special conditions obviously prevail in certain bog plants (65). In mesophytic plants the oxygen pressure is probably decidedly lower behind the endodermis than in the cortex, in which case the young, not yet suberinized, endodermis cells ought to have a similar pumping function as the epidermis. The old idea that the endodermis was endowed with the essential function of accumulation is certainly wrong. An analysis of the different parts of the root gives high salt concentration values for the cortex, and also for the meristems. The idea of the accumulation mechanism working in a protoplasm layer separating two media of different oxygen pressures can be applied to single cells as well as to tissues. The root cells are integrated in a falling oxygen gradient between the epidermis and the central parenchyma.

Heavy accumulation usually occurs in the cell sap of vacuolized cells. It seems questionable to assume an active secretion of salts from the protoplasm to the sap (53) as the concentration of cations at least is probably also high in the protoplasm (54). Furthermore, nonelectrolytes attain considerable concentrations in the protoplasm (55, 56). Even if the outer and the inner surfaces of the protoplasm show different properties, which has been recently demonstrated in *Nitella* (57), there is no reason to assume an active secretion into the cell sap. The accumulation in the sap is a reversible process (52). With inhibited absorption from the medium these numerous reservoirs are again emptied (5). Too little is known about the forces which transfer salts from the plasma to the cell sap. Considerable quantities of cations will "passively" be released by means of ion exchange from the vacuole membrane into a sap charged with carbon dioxide and organic acids. The mobility of the carbon dioxide and the metabolic instability of organic acids in the root cells (see above) may also induce an exchange of mineral anions. It seems probable that a dynamic nonpolar equilibrium prevails between protoplasm and sap. Related to the temporary storage of salts in the vacuoles is the question of the cell walls as transport paths. If the long-way transport through a multicellular tissue runs from protoplast to protoplast across the walls, these will stand under a considerable concentration pressure. As all walls constitute a continuous system, a certain "passive" transport of

salts through the walls is certainly possible and even probable, but owing to the thinness of the walls, the transported quantities cannot be of the magnitude that some investigators imagine (58, 59). The possibility, however, of such passive transport should be considered in discussing the possible influence of transpiration upon the transport of salts.

The quality and quantity of the salts which are exuded into the vessels of the root depends upon the salt content of the central parenchyma surrounding the vessels. The exudation into the vessels may be measured by the bleeding from decapitated plants. The salt content of the central parenchyma, on the other hand, is determined by centripetal transport maintained by means of the anion respiration. At inhibited salt absorption, e.g., in roots placed in distilled water, the cortex is impoverished with respect to salts, and exudation decreases. Deficiency of anions is of course most critical. The dominating anion normally being $NO_3^-$ a shortage of anions may easily develop through stimulation of the nitrate assimilation (proteinization) by means of an extra supply of glucose (5). The bleeding sap in this case receives an excess of cations ($K^+$), balanced by $HCO_3^-$ from the respiration. An excess of cations also appears after inhibition of anion respiration with hydrogen cyanide. This illustrates the independent transport of cations. Normally, the role of carbon dioxide as an anion is replaced by other, actively transported anions. A similar passive exudation of cations under anaerobic conditions is observed from the outer surface of the root, if the medium contains carbon dioxide. The migration velocity plays a deciding role in the qualitative composition of the bleeding sap under aerobic conditions. At comparable concentrations in the medium the rapidly moving $K^+$ and $NO_3^-$ usually dominate over ions such as $Ca^{++}$ and $SO_4^{--}$, whereas $H_2PO_4^-$ behaves intermediately.

The output of water is causally connected with the exudation of salts (5, 43). The quantity of water is inversely proportional to the osmotic value of the exuding cell. For this reason the central parenchyma exudes a diluted salt solution, when the osmotic value is low, e.g., at inhibited absorption (culture in distilled water) or a deficiency of easy moving ions. Correspondingly, ions which are absorbed from the medium must be accompanied by a quantity of water, which is inversely related to the osmotic value of the absorbing cells. Generally, the transport of osmotically acting sub-

stances, salts, glucose, carbon dioxide, etc., includes a simultaneous transport of water. Osmotic substances, e.g., carbon dioxide, appear metabolically on different points of the organism and entail *ipso facto* an attraction of water. Inversely the metabolic disappearance of an ion or a molecule, e.g., at the proteinization of glucose and nitrate, implies the liberation of a corresponding quantity of osmotically "bound" water. Should such processes occur in the central parenchyma, the "extra water" will be exuded in the vessels, thus diluting the sap, a phenomenon observed after treatment of the roots with a solution of glucose. The hydrostatic force of exudation is identical with the turgor pressure of the exuding cells, a fundamental osmotic fact which explains the high hydrostatic pressures observed (60).

This pressure may be varied by experimental modification of factors such as ion absorption, anion respiration, glucose supply, etc., which regulate the osmotic value of the central parenchyma and the balance between absorbed and exuded osmotic substance. The force of bleeding ($P_i$) can also be studied from an experimental variation of the osmotic value of the medium ($P_o$). The bleeding ceases at $P_i = P_o$ and the intensity of the bleeding (Bl) at varying $P_o$ was experimentally found to obey the formula $\mathrm{Bl} = \mathrm{k} \cdot (\sqrt{P_i} - \sqrt{P_o})$. Since $P_i$ is the sum of the forces which in the moment of exudation determine Bl, including the exudation of extra water, no simple relation prevails between the concentration of the bleeding sap and the concentration of the medium, but the root system can go on exuding a sap considerably more dilute or more concentrated than the medium (61).

These investigations on the bleeding phenomenon have been presented at some length because bleeding comprises the active part of that "sap movement," in absence of which the green parts of the plant would be deprived of mineral nutrition. The influence of the transpiration stream is still under discussion. We restrict ourselves to the following remarks. The overestimation of the passive transport through cell walls (58, 59) has already been emphasized, even though the inner walls of the root cells remain under a general "accumulation pressure" owing to the active transport from cell to cell. It seems improbable that salts will be sucked through cell walls directly from a diluted soil solution. Even halophytes, the roots of which dip in strong salt solutions, mostly contain surprisingly moderate quantities of sodium chloride. The

transpiration stream chiefly passes through the living cells of the root and the salt ions are then separated from the water, if they are not actively transported. The majority of the salts which are transported by the transpiration stream, originate from active accumulations in the lower part of the vessels. They represent a selection of salts, extracted from the medium. The transpiration stream no doubt accelerates the forwarding of these salts. At inhibited transpiration the salts remain in the sap, which probably retards further accumulation. Depending on the rapidly rising hydrostatic pressure, the sap is then pressed out through hydathodes, etc., a process, through which a certain supply of salts to the green parts is secured. A considerable "desalting" of the sap occurs in this case during its way through the plant (5, 50).

## Relations between the Roots and the Soil

The absorption of mineral salts by the roots is regulated by the aeration (oxygen, carbon dioxide) and the concentration of ions, including $H^+$. An important factor is the supply of glucose from the leaves. The mineral nutrition is consequently intimately connected with photosynthesis, which may explain why phosphate is more absorbed at day than at night (55). Maximum bleeding in daytime is perhaps also caused by the glucose factor. Cutting of the sieve tubes stops the downward transport of glucose, which is probably why absorption of mineral salts decreases after ringing (62). In cotton much of the decline in mineral uptake with heavy fruiting can be attributed to the reduced movement of carbohydrates to the roots (63). In defruited plants the uptake of salts rises. Exudation of salts through the roots at the end of the vegetation period possibly depends on similar circumstances. This is an illustration of the steady state between passive exudation and active accumulation. It may be recalled once more that "leakage" and "exudation" here mean outward movement of ions by ion exchange processes and not simple diffusion. The ions do not necessarily go out following a decline in the active accumulation process. The medium has to offer suitable conditions for ion exchange, too. It therefore seems to be inappropriate to conclude that "the mechanism responsible for the accumulation of salt is not the same as the mechanism which maintains the concentrations once accumulated" [(40], p. 70]. A slowness in the ion exchange can also depend upon the disappearance of active

groups in the plasma membrane. Outgrown cells with a nondissociated protoplasmic membrane ought to hold their salts with a minimum of metabolic support.

To what extent the observed increase of the root respiration in mycorrhiza and the simultaneous increase of the absorption of salts from clay (64) depends on a stimulation of the anion respiration, or follows from the intensified weathering induced by carbon dioxide cannot yet be decided. The quantity of carbon dioxide liberated by roots and by bacteria in the rhizosphere is certainly a powerful tool in the mobilizing of the cations adsorbed in soil colloids. The importance of the oxygen supply is equally great and it is significant that bog plants, such as rice and Nuphar, transport oxygen from the aerial parts to the roots (65, 66). As to the influence of $C_H$ and of the ion antagonism on the salt absorption from the soil no recent investigations are available [for an application of the law of mass action on the ion antagonism, see (67), p. 21]. The widespread occurrence of antagonism between $Ca^{++}$ and $K^+$ must not obscure the important fact that small quantities of $Ca^{++}$ are indispensable for normal conditions of the protoplasm, for wall formation, etc. This is probably why the absorption of potassium usually rises with the supply of very small quantities of calcium. The antagonism comes into play at somewhat higher concentrations. The hydrogen ion regulates to a certain extent the absorption of cations but is less active in the case of anions. This supports the theory of separate absorption of anions and cations and the metabolic conductance of the former. The mechanism of anion respiration is probably placed inside the protoplasmic membrane where the buffering is high [cf. (26) and (27)].

Exchange processes and buffering play an important role in the soil. The idea of a special "contact exchange" (68) between the root surface and the soil colloids is questionable, because the uptake of salts from a solution is an exchange process, too. The protoplasm of the root epidermis is separated under all circumstances from the soil colloids by the cell wall and the thin solution layer (69). The observed effects of colloids differ only quantitatively from the conditions in water cultures, but the velocity of the exchange processes may of course be considerably altered by colloids. These behave as temporary accumulators of ions, primarily cations, but also anions (70). No "contact exchange" apparently occurs in the absorption of phos-

phorus from clay water systems (89). By adsorption on the surface of the colloids, the ions of the soil solution are presented to the roots in a seemingly higher concentration but are directly accessible for exchange. This is due to the high acidity of the root surface (the fundamental factor) and the absence of measurable counterpressure from the side of the soil colloids (69). The advantageous effect of this accumulation capacity of the soil colloids is not utilized, however, unless the soil solution continuously transfers the ions. In the reverse case there will be a fixing of ions in a colloidal medium, in which diffusion proceeds only slowly (71).

Perhaps the soil colloids exhibit indirectly an advantageous effect as buffers against changes in the pH (72). The buffering capacity may profitably influence the availability of iron (73).

## Tissue Analysis as a Guide to Soil Fertility

The fact that the uptake of salts from the soil is influenced by so many factors makes chemical analysis questionable as a universal method of determining soil fertility. Current methods of soil analysis, by means of which a field is mapped ("Bodenkartierung"), depend on the assumptions that the roots are extracting the soil in a manner comparable to dilute acids and that the uptake of the dissolved salts is simply related to the concentration. Following these methods the soil samples are extracted with the aid of diluted, mostly buffered, solutions of acids and are then analyzed for potassium and phosphorus. The ability of the soil to deliver nitrogen depends chiefly on its microbiological activity and cannot be determined by chemical extraction only. The practical value of the chemical soil analysis varies in different countries. There seems to be at present a general trend to develop more appropriate physiological methods.

A critical comparison between soil analysis and tissue analysis (67) shows that the former conveys a rough average estimation of the available quantities of potassium and phosphorus, but the precision of the single case is not very high. *A priori* methods of a plant physiological pattern are logically better prepared to suit the purpose. As to the process of extraction, it is true that strongly acid substances act in the surface of the roots, but the actual acidity is a function of the ion assembly in the medium, too. Factors such as aeration of the soil and supply of glucose to the roots are by no means replaceable by chemical extraction. The ion antago-

nism, a phenomenon which largely regulates the uptake of potassium, calcium, manganese, and minor elements, is not revealed by chemical analysis only. A general drawback of soil analysis is the difficulty of representative sampling. The root system is more or less deep going and it is difficult to know in advance from which layer the main supply of salts is absorbed.

The plant physiological methods, known as tissue diagnosis, have been recently reviewed (67, 74). The fundamental thesis here is that only those nutrient salts which have entered the plant determine growth and fruiting. The analysis of the whole plant, even if performed at an early stage, is not to be recommended (67). A more reliable method is the choice of tissues, the nutrient content of which serves as an indicator for the mineral condition of the whole. Usually the leaves are chosen as being organs whose concentrations of nutrients most exactly reproduce the quantities of potassium, phosphorus, calcium, and nitrogen, which control the growth (foliar diagnosis). Comparative investigations on cereals have shown that the straw functions as a more unspecific container of nutrients and that the spikes and fruits show little variation as to the percentage of nutrients; the nutrient supply is here more likely reflected in the number of fruits. The leaves on the other hand through their central functions, photosynthesis and protein formation, have a deciding influence on the quantities of nutrients transported to the growing shoots and fruits. The nutrient content of the leaves thus reflects both the supply from the soil and the future yield. A practical method of foliar diagnosis can thus be built.

As to the most suitable time of sampling, investigations on cereals (67, 71) show that the highest concentrations are attained in an early stage of development, but that the period of flowering is more specific and recognizable. The percentage of potassium, phosphorus, calcium, and nitrogen, expressed in mg-atom per cent dry weight of the leaf powder, is sufficiently constant during this period of one to two weeks. It has been objected against leaf analysis that the nutrients can vary a great deal without corresponding variation of yield. A thorough investigation shows that this is true only at supra-optimal percentages. A distinct limit can be distinguished, below which growth inevitably decreases. Values below this limit are the only ones which have an interest from the viewpoint of fertilization. One of the advantages of the tissue

analysis is determination of the nitrogen (Kjeldahl-N), because this enables a judgment of the interaction of the different nutrient factors. The probable effect of fertilization with phosphate thus increases with the N-value of the leaves. Fertilization with potassium salts is in a similar way related to the values of phosphorus and nitrogen in the leaves, etc. Similar results will probably be obtained with corn (75), spinach (76), potatoes (67) and other agricultural plants [see the review (74)]. Trees and bushes, including vine, rubber, and lemon, have been investigated at an early stage of foliar diagnosis (77). It requires experience to choose a suitable time of sampling for different plants. It is fortunate that the variation is so small for cereals between leaves of different height on the straw [(67), p. 108]; here a general sample can be prepared from all functioning leaves. With other plants a certain selection may be more appropriate (74).

In special plants organs other than the leaves give more accurate or more convenient results. The roots of sugar beet are well adapted to tissue analysis. Samples of the stem give the best results for beans (76).

The practical purpose of the tissue analysis is to serve as a fertilizer guide for the coming year. The applicability of the results to the conditions likely to prevail is of fundamental importance. For example, it is important to know whether leaf analyses from samples, collected in a dry year, may be applied to conditions prevailing during a wet year. This has been investigated partly by means of pot cultures with varied watering and partly by taking samples from the same field during a course of years (67). The values of nitrogen and calcium generally vary more than the values of potassium and phosphorus; the latter are, on the whole, more constant. The inverse variation of nitrogen and soil moisture has also been demonstrated in leaves of trees (81). In summary, the results with cereals suggest that these variations do not seriously jeopardize the foliar diagnosis as a method of forecasting the effect of fertilization. A comparison between soil analysis and leaf analysis has resulted in a higher degree of reliability for the latter (67).

Because of the high economic values at stake in farming it is to be hoped that the method of tissue diagnosis will be given serious consideration. Tissue analysis, of course, is extremely well suited to trees with their widely outspread root systems. Fertilizer experi-

ments with trees are always tedious, and the taking of representative soil samples is rather illusive (78). Some years ago interesting investigations were performed, in which leaf analysis was used to determine the nitrogen nutrition of forest trees (79). Apple trees recently have been the objects of extensive investigations (80). Methods related in some degree to leaf analysis and mentioned in passing are "electrofoliar diagnosis" (82), injections of salts in leaves and observation of the effects (83, 84, 85), microchemical tests on the ash skeleton of leaves (86), and other more qualitative rapid methods (87, 88).

## Special Biochemical Effects of Some Elements

Distribution of the minor elements in soil and in plants has recently been reviewed in a series of monographs in *Soil Science*. Separate reviews are also published (90, 91). The boundary between the minor elements and the ten classical nutrient elements is slowly vanishing; thus iron may be considered as a minor element in line with manganese, the importance of which as a general nutrient element is proved. The following discussion is restricted to a few observations, which may aid in elucidating still incompletely known biochemical effects (92).

The diverse and often multilaterate action of the nutrient elements has been repeatedly emphasized [cf. (71)]. A proposed grouping in "structural elements," "biocatalysts," and "biogenerators" (93) is misleading, partly because nature often attributes several purposes simultaneously to each element, partly because the conception "biogenerators" is quite hypothetical and probably incorrect. The nonmetallic elements, especially carbon, hydrogen, oxygen, nitrogen, phosphorus, and sulfur, are chemical bricks in the organic matter of the protoplasm and the cell wall, but this group is not distinctly separated from the group of metallic elements, many of whose important reactions are ionic. As an example magnesium is partly fixed in important substances, e.g., chlorophyll, partly acting as free ion, and partly involved in enzyme actions.

As an example of multilateral effects of an element, which almost exclusively acts as free ion, potassium may be mentioned. Owing to its high mobility the potassium ion enjoys a favored position as a regulator of the ion balance in the protoplasm and in the cell sap. But it also determines, often in its antagonism to

calcium, in part the physical qualities of the protoplasm. Recently certain biochemical effects of the potassium ion have been observed. $K^+$ is said to exercise a specific accelerating effect on the phosphorylation of the adenylic acid system of the muscles (94). Potassium is believed to control the condensation of hexoses to sucrose and starch (95), possibly connected with phosphorylation processes, since these in turn may be concerned in glucose polymerization. The presence of $K^+$ in the tissue is also believed to facilitate the movement of carbohydrates from the point of synthesis to that of storage (96). There is perhaps some relation between potassium and ascorbic acid in tomatoes (97). The possible substitution of potassium with rubidium is still not settled (98), but the general occurrence of rubidium in plant tissues is not questioned (134). The partial substitution of one alkali ion by another was perhaps most intensively studied in the case of potassium and sodium. One hypothesis even postulates specific chemical linkages with organic molecules in the protoplasm [cf.(12)]. If this were the case, it might perhaps be assumed that similar elements cannot replace each other in specific linkages, but only in the general ion balance, such as in neutralizing organic acids.

Magnesium is incorporated in the chlorophyll molecule, and it cannot here be replaced by other metals. The same is apparently true of hemin. A substitution of the iron by manganese does not inhibit a linkage to peroxidase protein (99), but the product has lost its property as an enzyme. Magnesium is assumed to form nucleates in bacteria (20). A similar function is probably to be attributed to calcium, which as calcium pectate ensures the structure of young cell walls. This probably includes a buffering effect (33). With iron and manganese, the biochemical effects are chiefly due to the capacity for valence change. The activity of iron is utilized in the transference of electrons in specialized organic systems. In the case of manganese its place in organic molecules is still unknown. Redox activity must be taken into account [concerning the effect of manganese on reduction of nitrate, etc., see (100,101)]. A suggestion of different biochemical roles of iron is shown in analyses of chloroplasts (102). There is a distinction between the iron bound in the stroma, and that functioning in formation of chlorophyll. The former is "residual" iron, which cannot be directly transferred to "active" iron. Manganese can be partially substi-

tuted by iron in the reduction of nitrate (100,101). The intracellular antagonism $Fe \rightleftarrows Mn$ also plays a role (103), because these atoms mutually oxidize and precipitate one another. High content of manganese is thus frequently coupled with low content of iron (104).

Concerning the genuine minor elements, e.g., copper, zinc, boron, the active quantities as a rule are so small that any effect in unspecific ion balances is improbable. This does not exclude the possibility that the uptake of some elements, e.g., copper, follows an adsorption isotherm (unpublished experiments). Copper has been found to accelerate the autoxidation of ascorbic acid (105), for which organic substances with acid or coordinated groups (cystine, glutamic acid, etc.) are inhibiting (106). Ascorbic acid is believed to form a monophenol peroxidase with copper and iron ions (106). Active specific copper-proteins from a number of plant sources have been purified (107). With deficiency of copper, formation of nitrogen compounds is believed to increase at the expense of carbohydrate reserves (136). In the catalytic actions of copper, valence change $Cu^{+} \rightleftarrows Cu^{++}$ may play a role, which perhaps explains the interactions between copper and iron. Part of the action of copper (as well as that of zinc and manganese) in the soil may be through reactions affecting the oxidation of iron and hence its absorbability by the plant roots or its transport through the plant (107). Little is known about the poisonous actions of larger concentrations of copper, where more general ion actions are implied, such as denaturation of the protoplasm by heavy metals. More specific influences are also possible in poisons. Thus ribonuclease is said to be inhibited by copper and zinc (135). The viscosity of amoeba protoplasm is increased by copper (108). Here an antidote is bicarbonate (precipitation of $Cu^{++}$). Even if the presence of small quantities of copper is essential to normal life of a number of plants (91, 107, 109), the extent to which copper is an indispensable element to all higher plants must be regarded as undecided.

The same general judgment is valid for zinc. The thorough investigation of the biological tasks of zinc have suffered from the difficulty of finding sufficiently sensitive analytical methods. Spectral analysis has developed to a universal tool of researches for minor elements, but the high ionization potential of zinc and its concomitant weakness in spectral emission, especially in the pres-

ence of alkali metals, makes this element less sensitive for spectrographic analysis. By suitable arrangements of the electric arc, the sensitivity has been considerably increased, (110), so that more rapid advance may be expected. Biologically zinc creates a new interest by its presence in carbonic anhydrase. The content of zinc here amounts to 0.3 to 0.33 per cent (111). Zinc is said to co-operate in the protein synthesis (92) and in the stabilization of the redox equilibrium in the protoplasm. Earlier the relation of zinc to the formation of auxin and the appearance of certain diseases caused by absence of zinc were noted. A small quantity of zinc thus is required for the normal growth and fertilization of peas (112).

Investigations as to the necessity of minor elements are hampered by the very small quantities required, as well as by the difficulty of procuring absolutely pure chemicals and culture vessels. The seeds usually contain certain quantities of minor elements [cf.(113)]; it is often necessary successively to grow several generations. The external symptoms of deficiency are often indistinct, but generally imply a decrease in the activity of the meristems, a circumstance which points to general disturbances in the biochemistry of the protoplasm. In the physiological studies of the action of minor elements certain general phenomena were insufficiently emphasized, e.g., the different migration velocity of the ions in the tissues and the complications which arise from ion antagonism and from the secondary influence of the anion respiration on the migration of cations. Ion antagonism schematically means a decrease of the surfaces accessible for the transport of ions. Numerous earlier investigations have demonstrated the accelerating effect of fast moving anions, e.g., $NO_3^-$, on the transport of cations. Of the minor elements iron and also copper in this respect seem to be influenced by the nitrogen nutrition (138). Growth intensity can also give the impression of antagonistic phenomena (67); in rapid growth, slowly moving cations, e.g., iron and manganese, are left behind. All these circumstances contribute to the often contradictory results of pot cultures with minor elements. An additional factor is the varying solubility and availability of the elements in the soil. The availability of manganese is greatly influenced by the pH, the redox potential (139), and the antagonism of calcium. Specific plants accumulate zinc (114) and transmit it to the soil in available form. The results of the soil analysis are, as a consequence, less useful [on a comparison between soil and tissue

analysis with respect to zinc, see (115)], whereas tissue analysis should give more accurate results. In the latter case, however, a "luxury" storage of superfluous quantities, such as precipitates in roots, xylem, etc., must also be considered.

Investigations on the biological importance of nickel and cobalt, two geochemically related elements, have not been considered biochemically. The average accumulation of nickel is ten times that of cobalt, in spite of the similar concentrations of these elements in the soil (116). Molybdenum was earlier demonstrated as being essential for the normal development of *Azotobacter* and *Aspergillus* and positive results have also been obtained with higher plants (117, 118), but further information is needed concerning the role of molybdenum. The same is true of gallium (119) and rare earths (118), which accumulate in certain plants, e.g., hickory leaves. The well-known fact that different plants absorb nutrient and minor elements in varying relations and that certain plants accumulate preferentially single elements or groups of elements does not depend on a mythical "Wahlvermögen." An example of an element that has no nutrient qualities but is nevertheless accumulated in fairly large quantities is chlorine. The apparent favorable effects of chlorine on tomatoes, cotton, and peas (120), and the similar effects of sodium on beets, is not necessarily a nutrition effect, but more likely a substitution to maintain the proper ion balance in the cells. Even in a chemical linkage of minor elements in the protoplasm, substitution effects may be involved, e.g., concerning elements acting as coenzymes. Related elements may possibly produce similar substances which exert slightly different effects in stimulating or retarding metabolic processes. Under these circumstances it may be difficult to distinguish between "nutrient" and "stimulating" minor elements. It may be recalled here that manganese is shown to replace magnesium in fermentation. Chemically a substitution of related elements in organic linkage may be paralleled by substitution of related metals in the crystal lattice of geochemical compounds. Aluminum is among those elements which are occasionally taken up in large quantities but which do not exhibit significant physiological influences. Aluminum is heavily accumulated in *Lycopodium*, *Hicoria*, and *Symplocus* (119), is probably not absent from any plant. Its wide distribution is similar to that of silicon. Only pteridophytes seem to require aluminum as a nutrient, but the ecologi-

cal importance of this element is mostly due to its poisonous action in acid soils. There is often a surprisingly low uptake of this common element, depending probably less on its low solubility [(119), p. 29] than on the low mobility of $Al^{+++}$ or $Al(OH)^{++}$, the latter of which probably dominates in the soil solution. The low mobility of aluminum is demonstrated by the steep gradient that prevails in the concentration from roots to leaves. The physiological role of aluminum is frequently discussed, but is little known biochemically. The blue color of the petioles of *Hydrangea macrophylla* is caused by aluminum (121). With deficiency of aluminum the color goes over to pink; it is also influenced by the pH.

Among the alkaline earths, barium is a poisonous element, even in low concentrations (122), but a heavy accumulation of barium, apparently not injurious, is known in the Brazil nut (118). Strontium too exerts certain poisonous effects on the protoplasm (33).

Selenium has attracted attention, mostly owing to its poisonous effect on cattle (123). Selenium probably replaces sulphur in the proteins. It is accumulated in a number of species of *Astragalus*. Stimulating actions of low concentrations of selenium (2 mg. per kg. of sand) have been observed in millet, while greater amounts are poisonous (124). $SeO_3$ is apparently more poisonous than $SeO_4$. Toxicity in relation to the state of oxidation has also been observed in arsenic compounds. The nonmetallic elements whose roles involve chemical linkage are good examples of the consequences of the interchange of foreign element into the protoplasmic structure, which involve a nutrient element. The toxicity of arsenic may thus be due to a substitution with phosphorus and its influence is also dependent upon the amount of phosphorus in the medium. Similarly the deleterious effect of the chlorate ion may be caused by a substitution with the nitrate ion (unpublished experiments by B. Åberg). The possibility of substitution between cations, e.g., manganese and magnesium, iron and manganese, have already been mentioned.

The most important of the nonmetallic minor elements is doubtless boron (125). Its general necessity for normal development of a large number of plants is now well manifested. Additional studies on the interaction of boron and calcium have been made (125, 126). A specific relation may exist between boron and cal-

cium in pectin metabolism (137). It has however been assumed that the relation between boron and other nutrient elements is not determined by the type of ion (cation or anion) or the valence (127). The biochemical significance of boron has aroused numerous speculations. Investigations with spinach suggest that boron functions in the protein synthesis of plants (128), a somewhat indistinct statement that emerges also from studies of other minor elements. From chemically conducted experiments, the conclusion is reached that boric acid is combined with pyridoxine (polyhydroxy-compounds). It is assumed that boron co-operates in oxidation and condensation processes assisted by pyridoxine and riboflavin (129). Observations on the surface potential of the roots (130) suggest that boron is fixed in some way in the structure of the protoplasm. The fact that boron moves with difficulty within the plant has been ascribed to combination with large or immobile organical molecules (131).

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# GROWTH SUBSTANCES IN HIGHER PLANTS[1]

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## Introduction

This review deals with the work on auxin and related material principally with reference to the growth of higher plants and in the main covers the period since the last review by Van Overbeek in 1944 (1). Where necessary for clarity and continuity, reference is made to earlier literature, and to European papers published but not available in 1944. Not all phases of the subject can be comprehensively covered but it is hoped that the main results obtained and the major trends of development will be indicated. Other reviews and general presentations of the subject which have appeared recently or are in process of publication are by Went (2), Zimmerman (3), Bonner (4), Thimann (5) and Thomson (6).

Recent publications necessarily reflect the stress of the past five year period. There has been relatively less consideration given to theoretical aspects and new approaches, but rather an expansion of the work begun earlier together with great progress in the development of new active compounds and methods of their practical application to agriculture. Nevertheless, the writer feels justified, even at the risk of repetition, in devoting the main part of the review to the more fundamental aspects. The subject matter has been divided into three parts as follows: Methodology and the Types of Auxin in Plants, Mechanism of Auxin Action, and Auxin Effects.

A detailed discussion of terminology and suggestions for usage were given in the last review (1). The general confusion, overlapping and inadequacy of terms has been critically reviewed by Friedrich (7). Further comment seems futile until a firmer basis for distinctions in designations has been attained. The term auxin will be used in the generic sense for substances active in the *Avena* test and also for substances which fail in this test but have growth promoting activity in other tests together with molecular structure essential for activity comparable with that of indole-3-acetic

[1] Appreciation is expressed to Professor H. Burstrom for his interest and the facilities of the Botanical Laboratory, Lund, in the preparation of this manuscript.

acid (IAA). The term growth hormone will be limited to such substances which are also known to occur in plants. Intended meanings of terms in specific connections will be explained in the text.

## Methodology and the Types of Auxin in Plants

*Quantitative tests.*—The *Avena* test introduced by Went twenty years ago and later improved (8) is still the generally accepted standard test, although the modified, deseeded *Avena* test (9, 10) has been substituted in some laboratories. Judkins (11) has investigated sources of variability in the latter method including relations of size, age, position in the panicle and time of maturing of the seeds to sensitivity to IAA. Small increases were obtained with small and medium as against large seeds, with apical as against basal position in the inflorescence, no difference with age of the seed but a considerable increase with late as against early maturing varieties. The standard Swedish Victory oat is a late variety. None of the differences would explain the normal variability between individual test plants, but the exclusion of very large seeds seems practical. In the writer's experience the contact between the agar block and the coleoptile, i.e., the cleaness of the cut, the exact placing of the agar block and the amount of moisture at the cut surface, are the most important factors controlling uniformity of response. The question whether precursors as well as auxin contribute to the curvatures obtained in five hours in the deseeded test (12) has been answered in the negative in regard to the precursor isolated from corn endosperm (13). Some additional methods or modifications have been described (14 to 18). For the testing of the large and still rapidly increasing number of new substances exhibiting one or more of the many so called growth regulating activities the *Avena* test is of very limited applicability and other methods based on measurements of elongation, root formation, increase or decrease in cell number, shape or size of plants or organs, determinations of toxicity and evidently even death must be used in accordance with the particular effect under investigation. The specificity of these methods must be evaluated in each instance. The difficulties inherent in interpreting responses in curvature, epinastic movements, elongation of parts, variations in form and neo formation in plants in relation to the action of a particular applied chemical cannot be stressed too strongly. An

example will illustrate this point. Among recent methods one is the injection of aqueous solutions into one of the cotyledons of *Cucumis* seedlings and measuring resulting curvatures ten to twelve hours later (18). The method evidently gives a sensitive and quantitative response to IAA. However, large curvatures are also obtained with morphine, caffeine and evidently also with a large number of other mutually unrelated substances. Thus, at least with the modification of a preparatory dark period, curvatures are obtained with a large number of sugars and even glycerin (19). The latter are said to be active as a result of their direct utilization for auxin (Wuchsstoff) synthesis (19, 20) and the curvatures to be a measure of the rate of this process. On the other hand, measurements of elongation of stem regions of tomato plants supplied with sugars under essentially the same conditions have been used as a direct test for the presence of sugar in the elongating parts (21).

It must be concluded that the plant represents a delicately balanced system in which marked effects on growth and form may be obtained with a variety of substances in different concentrations including chemicals which are not specific in their action with regard to the final response but which have varying degrees of general nutritional or even toxic properties as well as by those which possess more or less well defined growth promoting activity. In fact, Guttenberg and co-workers (22, 23, 24) take the extreme position that even IAA as well as the entire group of synthetic growth substances of the indole, naphthalene and presumably also the phenyl series are not growth substances in the sense of auxin in the plant but are merely activators of the formation of true auxin in the tissues. This conclusion is based on evidence of two types: (*a*) the increase in auxin activity obtained in hot alcohol extracts from internodes treated with IAA lanolin paste, and (*b*) the decrease in magnitude and especially the delay in the time of appearance of curvatures in isolated and/or thoroughly starved *Avena* coleoptiles and *Helianthus* hypocotyls obtained with the application of IAA as compared with diffusates from tips and cotyledons respectively of these plants. With regard to the former, evidence of large amounts of acid stable auxin is presented, but it is not generally clear from the tables how far the increases obtained were of this kind. In one case refluxing with acid actually raised the yield above that of the untreated extract and occasionally inhibiting

material was obtained by refluxing with alkali. These facts greatly complicate the analysis. Referring the data to internodes and not to weight or volume may also be misleading after long exposures of tissues to IAA. With regard to the evidence from applications (22), the points might be raised that delay in appearance of curvatures was obtained even with coleoptile diffusates under certain conditions, that these diffusates were far from pure auxin A and most probably are at least in part IAA. In any case the results are striking enough to warrant further investigation, both from the above view point and with respect to the difference between "precursors" and active auxins which is based on time of appearance of curvatures (9).

*Solvent extraction and the forms of auxin.*—Our knowledge of the form in which auxin is present in plant tissues is intimately connected with the methods used for extraction. The two topics will, therefore, be considered together. Agreement seems to have been reached that auxin generally is associated with protein and the view proposed earlier (1, 25) that auxin may be classified under three main types namely bound inactive, bound active and a free form in equilibrium with the first two is borne out by the newer evidence so far as it goes.

Marked progress has been made in the development of rapid extraction methods with large yields. Avery *et al.* (26) have successfully applied the alkaline hydrolysis method, formerly used for endosperm, to green tissues simply by making the treatment more drastic. Yields of 0.03 to 0.035 mg. IAA equivalents per gram of dry weight have been obtained from leaves of cabbage and kohlrabi after autoclaving at pH 12 (1N NaOH) for thirty or more minutes at 15 lbs. pressure. Substitution of acid hydrolysis i.e., with 0.5 N HCl, also gave large even if lower yields. This is by far the most convenient and rapid method for obtaining large yields. The apparent simultaneous stability to acid and alkali is, however, difficult to understand. Shalucha (27) has applied the method to determinations of "total" auxin in peach buds at successive growth stages. She finds large and reproducible yields at all stages but still increasing with the rate of growth. Judkins (28) has compared different methods and solvents for the extraction of auxin from tomatoes. He finds alcohol extraction at pH 11.8 to 12 for eighteen hours at 26°C. to give the best yields. Van Overbeek *et al.* (29) report a method for the extraction of "free" auxin and

the release of large amounts of inhibiting material by heat treatments of sugar cane tissues. Large amounts of inhibiting substances as well as neutral inactive substances which may be activated presumably by aldehydases in the manner described by Larsen (30) have also been obtained by Hemberg (31, 32, 33) in the extraction of auxin from dormant and sprouting potato tubers.

Auxin has now been isolated in crystalline form and identified as IAA from corn meal (34) and immature corn kernels (35) by Haagen-Smit *et al.*, and from corn endosperm (36) by Berger *et al.* In each case about 10 per cent of the total potential activity was obtained as a crystalline product. Thus, IAA definitely occurs in higher plants but can hardly as yet be considered the only auxin present.

Information on the inactive bound form or precursor of auxin has become much more definite in several respects. Berger & Avery (36, 37) have isolated a stable auxin precursor from maize endosperm. It is inactive in curvature, root formation and swelling tests, but on heating at alkaline pH an active form is released which was crystallized and identified as IAA. A total of 43 mg. including *ca.* 10 per cent of a contaminant was obtained. This represents a 10 per cent yield of the total potential activity obtained by alkaline hydrolysis of the ground endosperm. Considering losses the authors estimate the major fraction of activity to be accounted for as IAA and over-all-yields of 30 to 40 per cent crystalline product to be attainable. The inactive precursor is relatively insoluble in water, acetone, ether, and absolute alcohol but soluble in dioxan and in aqueous solutions of alcohol, acetone, and alkali. Infrared absorption spectra and dialyzing properties are suggestive of protein or a protein complex, but its nitrogen content is only 4.7 per cent. Only slight yields of active material are released from this precursor with proteolytic enzymes of which chymotrypsin is the best. The isolated material, however, represents only a fraction of the original precursor, because *ca.* 40 per cent of the activity is removed by dialysis of cruder preparations as against only traces with the purified form. Wildman & Bonner (38) have isolated an auxin protein or auxin protein complex from spinach leaves, which yields auxin with either hot alkali or proteolytic enzyme treatments, but not with cold acid, alkali or by dialysis. This compound which is homogeneous, with a molecular weight of 185,000 and an absorption maximum at 280

mμ. is truly remarkable in that it constitutes 75 per cent of the total cytoplasmic protein and contains all the potential auxin activity of the plasma proteins. It exhibits phosphatase activity but is inactive with respect to all other of a number of enzyme activities tested and it contains no phosphorus.

Perhaps more indicative of conditions generally encountered in cells is the work of Gordon (39) on auxin protein complexes in wheat grains. He finds potential auxin activity associated with each of the five major protein fractions which have previously been freed from auxin by exhaustive soxhlet extraction with ether. Thus, tryptic digestion of the albumin (leucosin), globulin and proteose fractions from the embryo releases between 200 and 400 μg. and of the prolamine and glutelin fractions from endosperm between 25 and 100 μg. IAA equivalents per kg. Controls without enzyme give no activity. Autoclaving at alkaline pH releases auxin from prolamine, glutelin and albumin but is ineffective for globulin and proteose. Furthermore, both alkali stable, acid labile and acid stable, alkali labile auxins are obtained. Both types are present in the ether extracts from the grain and probably are both released from prolamine, glutelin, and albumin. Only the alkali labile type is released from globulin and proteose. Evidence obtained from electrodialysis indicates that the auxin is largely adsorbed on to the protein rather than a constituent part of it, as no activity is obtained after electrodialysis except in the case of gluten in which yields one-third as much as after ordinary dialysis. The lack of proportionality between rates of protein hydrolysis and amounts of auxin released is presented as supporting evidence for adsorption. The above work clarifies to some extent the sharp disagreement in yields obtained earlier with enzyme treatment of green tissues (40) and alkali treatment of endosperm (41). It indicates that in both cases the bulk of the bound inactive auxin is in some manner associated with protein, but it remains a question how far auxin released by enzyme, acid and alkali hydrolysis is derived from the same or at least in part from different, perhaps mutually exclusive precursors.

Tryptophane may be converted to an auxin active on *Avena* (9). This change occurs to some extent on standing in aqueous solution (42) and is accelerated by alkaline treatment (37, 43). The isolation of "auxin-proteins" and of crystalline IAA from plants reopen the question whether tryptophane is the principal auxin

precursor in plant tissues. Wildman *et al.* (44) injected tryptophane into spinach leaves and 3.5 hours later extracted ten times the amount of auxin normally present in controls injected with buffer solution. The conversion of tryptophane was also obtained *in vitro* with a lyophilized protein preparation, the responsible enzyme of which is confined to the cytoplasm of the leaf cells. Infiltration with indolepyruvic acid also led to auxin formation, suggesting this compound to be intermediary. However, the protein preparation does not attack indolepyruvic acid nor does it activate either indole ethylamine or indole acetaldehyde, which serve as precursors (9, 30). They suggest that tryptophane is the principal precursor and IAA the principal auxin in spinach leaves. In the case of the precursor from maize endosperm and the protein fractions from wheat grain this possibility has been ruled out because tryptophane would be inadequate in quantity present and extent of conversion by alkali treatment, to account for the observed activity, as well as, in the latter case, the improbability of its giving rise to acid stable auxin. It may be concluded, therefore, that tryptophane serves as a precursor of auxin and possibly as a main ultimate source of IAA, but it is not possible at present to consider it the principal precursor of auxin in tissues and generally perhaps not even the principal direct source of IAA.

A neutral growth substance, "skototonin," has been found in ether extracts from a variety of plants (30, 33, 45, 46, 47). Larsen (30) has made extensive comparisons of physicochemical properties and physiological activity of this substance with indole acetaldehyde prepared from tryptophane in two different ways. He found the two identical with respect to molecular weight (diffusion rates in agar), conversion to active acid form by aldehydrase (i.e., by soil, raw milk, and purified Schardinger enzyme from milk), stability to alkali and inactivation by acid. Neutral extracts from dark grown plants have higher activity than from light grown plants. Since the "variation" parallels the rate of elongation, Larsen considers it strictly as a hormone and as responsible for the increased growth in darkness. Larsen, therefore, considers it to be strictly a growth hormone. (This would be the first compound without a terminal carboxyl group showing activity as such in *Avena*.)Against this interpretation are the findings by Gustafson (48) that the auxin content generally is higher in darkened and nonchlorophyllous tissues and most significantly the author's own findings [(30)

Tables 9, 10A, 10B; Figures 14, 18], which show clearly that no trace of activity may be given by concentrations of the neutral substance which upon conversion to acid by soil treatment give high activity. Curvatures from the neutral fractions, therefore, might be explained by a partial conversion to acid. This might account also for the otherwise inexplicably low maximum angles on the basis of competitive inhibition by the neutral form (49). Considering the variable behavior of the nonconverted neutral fractions in the *Avena* test the degree of precision achieved in the molecular weight determinations is remarkable. Larsen computes the quantity of neutral growth substance (indole acetaldehyde) to be generally low in tissues but in cabbage to be at least 4 mg. per kg. fresh weight. Linser (46) considers the neutral fraction here to be the main one, between 50 and 300 mg. per kg. Even the minimum value equals the entire activity obtained after alkali treatment (total extraction), i.e., 30 to 35 mg. IAA equivalents per kg. dry weight or roughly 3.5 mg. per kg. fresh weight. Apparently the neutral growth substance may be one of the main "low molecular precursors" encountered in extraction work.

In spite of improvements in extraction methods the fundamental difficulties in relating yields of auxin to rates of growth or other physiological activity in the tissues still exist. It is generally agreed that the bulk of the very large yields that may be obtained by exhaustive extraction and conversion to active form has no direct relationship with the existing growth rates of the tissues. Some degree of correlation has frequently been found between diffusible auxin and growth rates of tissues exhibiting polar growth and situated immediately below the regions analyzed. Much effort has been spent, therefore, to develop extraction methods for "free" rather than "total" auxin and in attempts to distinguish between the two. On the basis of data available in 1943 Van Overbeek (1) calculated that in all cases the "free" fraction is only between 2 and 10 per cent of the total yields. Newer data for corn endosperm (37) indicate *ca.* 30 per cent to be in water soluble "free" form and Van Overbeek *et al.* (29) show that it may rise to 50 per cent in the lower halves of bean internodes after geotropic stimulation. Without distracting from the significance of these findings a few words must be said about the nature of the general problem. Assuming that present methods of extraction are capable of giving total yields of active and potentially active auxin from tissues so that a basis

exists on which to compute the "free fraction," then we are concerned with what constitutes the "free" form. It has been understood (40, 51) that the proportion which would exist in tissues in free form at any given moment would not be exclusively or even quantitatively represented by the values obtained from short time extractions, as variable amounts of loosely associated auxin and active material set free from the more stable forms would be included. It was also clear that the treatments required for extraction, even the least drastic, would affect the amounts that would be liberated. We now know that auxin complexes are of several distinct types. The concentration of free auxin in equilibrium with each of these must be different and in a mixture must depend on the types and proportions present. Their "dissociation" must occur not only at different rates but at rates differently affected by the conditions prevailing during the extraction. In an attempt to minimize influences from the extraction treatments Gustafson (52) has applied a method of quick freezing, drying and heating and subsequent extraction of the dry ground material with organic solvents, which is no doubt the best procedure developed so far. The possible objection may be raised, however, that as in the case of *Lemna* (53) large quantities of IAA added to the tissues immediately before drying may be fixed and are not released by ether except in the presence of water. Possibly other solvents are effective. It appears to be an error, therefore, to speak of extraction methods for "free" auxin and values for the fraction of "free" auxin can have no real meaning. Furthermore, the indications are that so called "total extraction" methods yield only certain portions of the potential activity and may vary in effectiveness from one tissue to the next. It may be concluded, that correlations between yields and growth still can be made only on a relative basis with closely comparable tissues.

*Synthesis of auxin.*—On the basis of various evidence the conclusion has been reached (54) that the synthesis of auxin, i.e., auxin precursor, takes place exclusively in the light presumably in connection with photosynthesis. The first demonstration of auxin synthesis in nonchlorophyllous tissue independent of light was by Nagao (55) who used cultures of excised roots growing on mineral deficient and complete media and this has been confirmed (56). Tissue cultures of *Nicotiana* similarly synthesize auxin both in light and in darkness (57). In all these cases auxin was obtained

only with extraction, but previous failures to obtain auxin in darkened plants are based on diffusion methods. The relation of auxin synthesis to light, therefore, must be in part the need for carbohydrate (19, 20) but also it seems probable that light plays a part in releasing auxin from a bound form.

The relation between auxin content and nitrogen nutrition has been further studied by Avery & Pottorf (58, 59). They show that nitrogen limits the formation of auxin only when supplied in the range between 0 and 0.1 p.p.m., which is much lower than the requirement for optimum growth. The auxin content of tissues may be roughly correlated with the total nitrogen content but need not be related. Thus, tetraploid and diploid kohlrabi were found to have approximately equal nitrogen contents but the latter yielded from two to three times as much auxin.

*Inactivation of auxin.*—It is well known that auxin is inactivated relatively rapidly in plant tissues whether or not it is utilized in the growth process (60. 61), Various factors such as light, especially in the presence of pigments, and oxidizing agents greatly accelerate the destruction. It is of interest, therefore, that such strong reagents as hydrogen peroxide and iodine have been used successfully in separating inactive auxin precursors from active auxin in plant extracts (46). This indicates that the bound form is protected in some manner from chemical attack. On the other hand, in the case of the precursor isolated from corn endosperm, a considerable fraction of the potential activity is lost by this procedure (37). Special importance has been ascribed to oxydizing enzymes in the inactivation of auxin *in vivo* (62, 63, 64). One such enzyme which apparently specifically attacks IAA has now been isolated from etiolated pea epocotyls by Tang & Bonner (65). It is strictly aerobic, utilizes one mole of oxygen and releases one mole of carbon dioxide per mole of IAA inactivated. It attacks the side chain but not the indole nucleus. It does not react with a number of other auxins or auxin-like substances which were tested such as indole carboxylic, -propionic, or -butyric acids nor with indole acetamide, tryptophane or naphthalene-, naphthoxy-, or phenoxy- acetic acids or their derivatives and none of these affects the rate of inactivation of IAA *in vitro*. The reaction is, however, inhibited by KCN (50 per cent by $10^{-4}$ mols l.) and by carbon monoxide in the dark but not in the light. This suggests

that the enzyme may be a heme protein. The optimum pH range is 6.2 to 6.7. Impurities in old crystalline preparations and inactivation occurring on standing especially in aqueous solutions have been (shown by Algéus (67) and others to be important) not only because of loss in activity, but also because the decomposition products exert inhibiting effects.

## Mechanisms of Auxin Action

Elongation of plant cells ultimately is caused by water uptake. It is well known that mechanisms for the action of auxin in this process have differed fundamentally as to whether the effective suction pressure required is produced by an action of the auxin in the cytoplasm which would eventually result in increasing the osmotic concentration and thus increase the suction pressure, or whether the auxin more directly affects the wall in such a manner as to decrease its resistance to extension. The latter would lead to an increase in suction pressure without the need for an increased osmotic concentration.

In a recent survey Thimann (5) emphasizes the former very simple mechanism in terms of increase in suction pressure leading to water uptake resulting from increased salt absorption which in turn is brought about by an influence of auxin on respiration. Thus, he utilizes essentially the evidence and interpretation advocated by Commoner *et al.* (68, 69). This mechanism which in its general features has been a latent possibility for the past ten years has been difficult to substantiate and pertinent objections to it are presented in the last review. Auxin may affect the movement of both solutes and water but a causal relationship still is not firmly established. Not only the effect of increasing the uptake of water in the absence of an external supply of solutes (1, 70, 71) but the "loosening of the primary wall" also obtained with IAA and occurring before commencement of elongation (72) must either be disposed of or taken into account. One feature of this mechanism, namely the linking of the four carbon dicarboxylic acid respiratory cycle to ion absorption, has been investigated by Machlis (73). He shows that additions of these acids or citrate reverse the inhibition of respiration and salt accumulation by iodoacetate and of accumulation but not of respiration by malonate. However he does not conclude that the respiratory system affected is specific for

accumulation. The work on the effect of auxin on respiration has been questioned by Avery (74) but is now confirmed in part by Berger, Smith & Avery (75). They obtain increases of 35 per cent or more in oxygen uptake with 10 mg. per l. IAA in coleoptile segments in sucrose solutions but they find malate, and therefore, presumably also fumarate, etc., to function as a substrate rather than as a catalyst in this reaction. This agrees with Lundegårdh's conclusion that fumaric and maleic acids may be intermediaries but not catalysts in respiration of wheat roots (76). However, Berger & Avery (77, 78) have found both the malic- and alcohol dehydrogenase enzyme systems isolated from coleoptiles to be stimulated by pretreatment of the tissue with IAA but not by addition of IAA to the isolated preparations. The conclusion may be reached that auxin plays a role in respiration. How far its effect in this connection is through a catalysis of the four carbon acid system and affecting salt absorption is still unsettled. Similar conclusions have been reached by Bose *et al.* (79).

A new approach to the mechanism of auxin action has been made by Burström (72) based in large part on measurements on individual cells. From work on osmotic relations (80), carbohydrate and mineral nutrition (81) and formative effects of carbohydrates (82) and of salts (83) in the growth of roots he obtained evidence for the separation of the growth of the cell into two more or less distinct phases. The first phase is characterized by a loosening of the primary wall, containing pectin as a main constituent, possibly without addition of new material. During the first phase the elasticity of the wall (per cent increase in length over the length of the plasmolyzed cell) and the turgor tension (the corresponding absolute increase in length of the wall) both increase to reach maximum values simultaneously at the end of the first phase. This phase is temperature-sensitive but rather independent of nutrient conditions. The rate of growth is slow. The second phase is characterized by constant turgor tension and decreasing elasticity. It is the period in which oriented cellulose strands are deposited in the wall. It is shortened by increasing temperature and extended by carbohydrate supply. It is of short duration, but two thirds or more of the total elongation occurs in this phase. A comparative study was made of the effects of IAA added to the nutrient solutions of intact and excised root cultures of wheat.

Observations were made on the growth and various physiological activities of the roots as a whole and also of the individual cells of the epidermal layer. The results agree with earlier findings as to the increase in elongation obtained with very low concentrations and the inhibition with higher ones, with however, the important difference that all concentrations, even the lowest, in the range from $10^{-6}$ to $10^{-11}$ mols per l. are at first inhibitory. The inhibition may be more than compensated subsequently by more than normal elongation in the low IAA concentrations. Thus, a treated root which after a given time may have grown exactly the same amount as the control and macroscopically would be indistinguishable from it, nevertheless exhibits regions at some distance from the apex with longer than normal cells followed by a region of cells shorter than normal. Inhibitions amounting to 80 per cent decrease and stimulations up to 50 per cent increase in length of the normal cells were observed. The first phase of growth of the cell, i.e., the increase in elasticity or loosening of the primary wall is consistently accelerated by the addition of IAA, even by strongly growth inhibiting concentrations, from values of 15 per cent in the controls to 20 per cent in treated plants. The inhibition of growth of the root cell is due entirely to shortening or complete elimination of the second phase. Advantage was taken of the parallel development of atrichoblasts and trichoblasts to determine the effect exerted on different parts of individual cells. With respect to stimulation (the first phase) the atrichoblasts are affected first and most strongly. The proximal part of the trichoblast is affected the least and is often inhibited, even when the cell as a whole finally becomes longer than normal. With respect to inhibition no differential effect was observed. No effect was obtained on the rate of cell division. A slight decrease in the number of longitudinal divisions was compensated for by a corresponding increase in radial divisions, which thus contributes to the increase in diameter of treated roots. Measurements of rates of inversion of sucrose (a measure of physiological activity of roots), changes in carbohydrate contents and glucose absorption in air and in nitrogen gave no significant differences with IAA treatments referable to the differences in growth. The conclusion is reached rather that the carbohydrate metabolism proceeds normally even during the periods of growth retardation. This is not to imply, however, that

growth is independent of respiration as a prerequisite condition.

The proposed mechanism of auxin action can be extended to account for the marked inhibition and subsequent stimulation of root growth (after effect) obtained in treatments of seeds with high concentrations of IAA (84). Furthermore, it is ultimately in complete agreement, though arrived at on quite independent grounds, with the mechanism proposed by Went (85) to account for growth and also hypertrophy leading to inhibition in stems. The only difference is in concentration range of applied IAA ($10^{-11}$ to $10^{-6}$ as against $10^{-4}$ to $10^{-1}$ M) which is also characteristic of other effects in roots and stems (86). The above mechanism does not specify the manner in which auxin increases elasticity during the first phase and in excess prevents the deposition of cellulose in the second phase of growth of the cell, but it is assumed that the same reaction on the part of auxin is responsible for both effects. Although Ruge (87) would associate the action of the auxin directly with the colloids of the wall, Frey-Wyssling (88) claims that the amounts involved are too small. Burström (80) accepts the view that auxin may play the role of a coenzyme. The seat of activity must then probably be in the cytoplasm or its surface layer bordering on the wall and specifically in processes concerned with polysaccaride transformations or synthesis. These must be processes involving relatively small expenditure of energy. Very likely, therefore, the respiratory processes supplying the bulk of the energy release (carbohydrate and organic acid degradations) may not be primarily concerned, although no doubt auxin action must ultimately be conditioned by the over all respiration and, vice versa may ultimately affect its rate. The work of Thimann and Sweeney (89, 90) showing that growth and auxin action in protoplasmic streaming are associated with only a small part of the total respiration as well as the work of Berger *et al.* (75) agrees with this view. Thus, it may be seen that the formerly quite unrelated if not contradictory evidence for the action of auxin on the wall versus in the cytoplasm is now beginning to fit into the same scheme.

Eyster (91, 92, 93), on the other hand, interprets the action of auxin in terms of an effect on the release of diastase from a bound to an active form. Veldstra in two papers (94) [not available to the writer but quoted in (95)] suggests that auxin affects permeability of the plasma membrane by occupying specific positions in the double layer or interface between water and lipids.

Thus far the mechanism of auxin action has been discussed in terms of effects observable as a consequence of its action. An attempt will now be made to analyze the more dynamic aspects of the auxin reaction. Very little is known about the reaction system of which auxin is a part or about substrates that are directly involved. Evidence may be considered sufficient to state that oxygen is required, that sugar is ultimately needed and that eventually respiration is affected as discussed above. This would indicate that auxin may play the role of a coenzyme in a manner similar to that which has been demonstrated for some of the vitamins. Considerable evidence can be brought to support this view even though none as yet constitutes conclusive proof.

*Chemical structural requirements for auxin activity.*—One of the strongest arguments for a specific chemical reaction on the part of auxins generally is based on the essential structural requirements common to all molecules with auxin activity. These have long been considered to be a ring with a side chain containing at least one carbon atom in addition to a terminal carboxyl group, a double bond in the ring adjacent to the side chain and a certain spatial relationship between the double bond and the terminal carboxyl group. Additional structural features of the molecule may affect its reactivity, transportability and stability but are not essential for activity. Three findings, the reported activity of indole acetaldehyde (30), the activity of halogen and/or nitro substituted benzoic acids in a number of tests on green plants (96) and the activity of naphthalenenitromethane (95) would seem to require a modification in the minimum essential requirements. On closer examination, however, the above cases may be the type of exceptions that prove the rule. Larsen's data suggesting that the activity is related to the conversion to acid would make indole acetaldehyde comparable with the indole acetic acid esters whose activity is a function of the extent of hydrolysis (97). With regard to substituted benzoic acids which may be active, on the other hand, it is known that the introduction of groups into the ring will affect the position of the carboxyl group. This may cause it to shift with respect to the double bond into a position more nearly like that of the carboxyl groups of longer chains so as to increase the reactivity. More definite information on this point should be obtainable from x-ray data. The activity of naphthalenenitromethane the authors ascribe to the "aciform," $-CH=NOOH$,

which differs from active napthaleneacetic acid only in the substitution of *N* for *C* in the carboxyl group. Although the specific reaction of the auxin molecule is unknown, the aerobic nature of auxin action together with the indispensability of the double bond and its exact position in the ring suggest that it may be simply the reversible saturation of this double bond. The assumption that auxin is a coenzyme implies further that it is associated with a protein (apoenzyme) and through it with specific substrates. No such active auxin protein complex has been identified. It is not likely to be any of the relatively stable auxin yielding proteins or protein-like precursors which have been isolated. It may be deduced that the system with which auxin reacts must under normal conditions be rather limited in quantity because more auxin is obtainable by diffusion from rapidly growing plant tissues than is required for optimum rates of growth. As suggested by Went the proportionality between concentration applied and rate of growth over a considerable range indicates a high degree of association of auxin with its reaction system. The sharp break at the maximum response after which no increase in rate occurs with increasing concentrations must on the other hand be determined by the degree of association of the substrate with the enzyme complex or its utilization as in the original "food factor" interpretation by Went and by Dolk (54, 98, 99). It appears, therefore, that the amount or degree of saturation of the auxin reaction system, in which substrates or specific proteins as well as the concentration of auxin *per se* may be limiting, determines the rate of auxin action.

*Interaction of auxins and structurally related compounds.*—The conclusion just stated has been arrived at also on different grounds (100). Experiments showing competitive inhibition of IAA by less active compounds with auxin structure and an evaluation of available data on relative activity of different auxins in promotion and inhibition of growth in various tests led to a scheme explaining the effects of auxin action in terms of quantitative functions of two kinds of properties of the molecules. The first property is a specific chemical reactivity due to a specific reactive group in the molecule and modified in degree in different compounds by other groups present. The second is a specific chemical structure permitting the molecule to occupy a specific position in a large molecular aggregate. This aggregate was considered to be a protein-auxin-substrate complex, in which the protein represents the

apoenzyme and auxin the coenzyme in what may be termed the auxin reaction system. According to this scheme the activity of auxin in growth promotion would be dependent on both the degree of its chemical reactivity, i.e., affinity for the substrate, and its molecular configuration, i.e., the degree of its association with the apoenzyme-substrate complex. The inhibiting action on the contrary would depend only on the second property which determines the extent of association between the molecule and the complex or its separate components. Not only the affinity of the compounds for the apoenzyme of the active complex but also their affinity for the protein complexes representing bound inactive types would be important in that compounds with higher affinity for one or more of these proteins would effectively replace the auxin. The resulting rapid auxin release might cause a temporary stimulation and even abnormal growth, but would, however, ultimately lead to a loss of auxin and in extreme cases would result in the complete destruction of the normal auxin supplying mechanism. It is suggestive that the action of highly effective weed killers may be of this type. In fact, in accordance with Guttenberg's views (22) all synthetic auxins, including IAA, would come under this group. The degree of activity and/or toxicity of the compounds then would be a quantitative function of the degree of their affinity for specific proteins. A striking example of the type of effect discussed above is the relation of biotin sulfone to the activity of biotin (101). The sulfone is inactive but by replacing biotin from avidin small concentrations actually promote growth whereas higher concentrations are inhibitory. In so far as this type of activity would not involve chemical reactivity it is to be expected that a variety of compounds with only partial auxin structure would be effective. Growth inhibiting compounds are of special interest in this connection. The inhibitor active in *Avena* isolated by Stewart (102) is convertible to auxin. Similarly the only crystalline preparation of the inhibiting agents secreted by guayule plants was identified as cinnamic acid (103), the *cis*-form of which is active as auxin and the *trans*-form inactive. Furthermore, a number of growth inhibiting substances isolated from ripe fruits and other sources on the basis of their function in inhibiting seed germination and root growth have partial or incomplete auxin structure. Para-sorbic acid and related compounds as well as coumarin are highly effective (104, 105, 106). The importance of lactone structure is stressed.

Veldstra & Havinga (95) who have also observed the similarity in structure between auxins and these growth inhibitors further show that digitalin, benzocoumarin, angelica lactone and lumi auxin are of this type. They point out that both auxin a and b after formation of the lactone ring (which is known to occur), and in the case of auxin a upon removal of one molecule of water, would have structures characteristic of the growth inhibitors. If the unsaturated lactone ring be an essential requirement for inhibitory activity, the inhibition of growth resulting from high concentrations of active auxins could be explained by a partial conversion of the compounds into lactone or intramolecular rearrangement to the required structure rather than to a mere super saturation of the auxin reaction system. Snow's interpretation of bud inhibition (107) postulating the formation of an inhibitor in the presence of auxin in the tissues would be completely substantiated on this basis. The reviewer prefers at present to consider both alternatives as possible, concurrent and not mutually exclusive. Clearly many of the inhibitory substances are of wide occurrence. Compounds of the benzaldehyde and fagarine types (108) might also be considered, and the recently reported effects of benzene on mitosis and on root growth may be pertinent (109). They may be considered as buffers regulating the sensitivity of response of tissues to auxin in the same manner as inactive sterols regulate the sensitivity of animal tissue to the active sex hormones as shown by Lipschütz (110).

*Stimulation versus inhibition of growth and the regulatory function of auxin.*—The above scheme accounts for stimulation and inhibition of growth as quantitative expressions of a single effect and the two cannot be readily separated. Stimulation by auxin may be brought about in different ways. In cases of deficiency, increases in the free form will correspondingly increase the associated active form and thus to a point promote growth. The increase in the free form in turn may result from several causes, most likely by conversion or release of precursors of different types rather than by a direct synthesis as discussed above. Inhibition of growth results from oversaturation of the auxin reaction system by low activity auxins, excess active auxin or by substances with partial auxin structure. A second stimulation may follow such an inhibition by a temporary association of the excess auxin or the inhibitors with

the specific protein and/or substrate thus effectively raising the concentration level of the auxin reaction system and therefore also of the auxin level permitting promotion of growth, i.e., an adaptation to increased auxin concentration occurs (72). In so far as this increased activity would be reflected in a withdrawal of substrate from adjacent tissues it would represent a stabilized increase in rate of growth of one region or organ at the expense of others (a correlative effect) and in so far as it would affect the absorption of limiting nutrients it would represent an increase in rate of growth of the plant as a whole. The latter would hold particularly with reference to unicellular and other small plants, in which auxin-induced increases in rates of multiplication need not be interpreted in terms of an effect of auxin directly on the process of cell division.

The above type of scheme has been fruitful in enzymology and has been used successfully to clarify the action and interaction of some vitamins and their homologs (vitamers). None of these systems, however, offer the wide range of known compounds or variety of effects on growth that may be obtained with the auxins and proper choice of concentrations, conditions and plant material. Its use in attempts to survey common and essential features of different compounds and plant responses appears to be helpful not only for classifying known information but in suggesting new approaches and is, therefore, justifiable now regardless of its future possible inadequacy. In this connection the question may be raised as to the importance of auxin in comparison with other substances in the control of growth. Certainly several of the B vitamins and possibly all the members of the bios group are essential, carry out specific reactions and exercise rigid control of the rates of the particular processes of which they are a part. In the higher plants, however, they appear to be normally present in larger than required amounts (111 to 114) so that an effect on growth is obtained only under rare conditions of extreme deficiencies. Perhaps still more important, the systems which they regulate constitute the more general energy-supplying metabolism, so that a variation in any one vitamin is not readily translated into visible morphological effects. The significance of auxin as a hormone or growth regulator in the higher plants then would lie first in its function in a specific part of the respiratory system

which is intimately connected with the synthesis and/or transformation of cell wall constituents and secondly in its minute quantitative requirements, extreme lability and transportability. These properties make it a highly sensitive mediator for the control of the growth process in response to many types of even minor changes in conditions both without and within the plant. The effect produced by auxin is not determined by its concentration nor by the rate at which it is released in active form (44, 89, 115) but more directly by the equilibrium between the auxin reaction system and any number of other systems supplying energy and substrates for synthesis within the cell. In organisms built on different structural bases, such as the fungi and bacteria, auxin may be essential as judged by its universal presence, but it does not exert a corresponding morphogenetic function. Evidently this role is played at least to a considerable degree by biotin and thiamine in the lower forms (116, 117, 118).

## Auxin Effects

Effects obtained with auxins and related substances cover the range of normal and abnormal development. Certain compounds are more effective than others in eliciting a particular response. This need not imply fundamentally different actions on the part of the different substances but rather different quantitative degrees of reactivity, stability and accessory properties adapted to the conditions and milieu in which the action is exerted. The material presented below on different auxin effects is considered from this view point.

*In growth of lower plants.*—The presence of auxin has been demonstrated in practically all types of plants but studies of its effects on the lower forms mostly have been cursory.

The effect of auxin on *Chlorella* has been investigated by several workers. Some report an increase in the rate of cell division, others an increase in cell size and still others find only inhibitory or no effects. A thorough study of the nutrition of ten species, including four *Chlorella* and five *Scenedesmus* species by Algéus (67) substantiates the stimulating effect on the rate of multiplication and gives good evidence for earlier discrepancies in terms of inactivation, inhibiting action of decomposition products and variability between species. Ascorbic acid in high concentration (1 gm. per l.)

also increases the rate, but lower concentrations are inactivated too rapidly to be effective. Ascorbic acid is shown to promote chlorophyll production rather than vice versa as has been postulated. Thiamine on the other hand seems to be present in optimum amounts as no significant increases resulted from its application. In *Ulva lactusa* IAA added to the medium especially in presence of manganese stimulates the early stages of development but does not affect the size or shape of the cells. Thiamine and ascorbic acid also stimulate growth, Kylin (119).

A species of *Calypogeia* contains auxin and responds to applied IAA by increased rate of cell division, but the final size or shape of the plants is not affected; Witsch (120). Seidl (121) has extracted auxin from *Selaginella Martensii*, a plagiotopic form, and has shown that the development of shoots, roots and rhizome from segments of the latter are under the control of auxin and may be varied at will by application of IAA.

*In tissue, single cell, and embryo cultures.*—For present purposes tissue cultures may be divided superficially into two types, those developed by White (122, 123) from tumors or tissues potentially liable to form tumors, and those by Nobécourt (124) and Gautheret (125) from cambial tissues. The environmental, mineral and growth factor requirements for rapid rates of growth of *Nicotiana* and *Helianthus* cultures of the first type have been further studied by Hildebrandt *et al.* (126, 127). Generally glycine and thiamine and often nicotinic acid and pyridoxine are included in the medium as beneficial for growth, but an essential requirement for these substances has not been established. They also tested the effect of added extracts from galls, yeast and bacteria (128). Some were stimulatory in small concentrations but often inhibitory especially in higher concentrations. An extract from Paris daisy crown gall was an exception in that all concentrations used stimulated the growth of *Helianthus* but not of tobacco cultures. Several organic and amino acids were found to be strongly inhibitory. With respect to auxin, tobacco and presumably other tumor-derived cultures produce sufficient amounts for optimal growth and tobacco cultures also produce an auxin inhibiting material (57). Addition of auxin to the medium is not essential but may markedly affect growth and composition. Fifty per cent increases in total nitrogen content per unit dry weight have been

obtained both with addition of IAA and naphthaleneacetic acid (unpublished). Cultures derived from normal cambium on the other hand apparently are incapable of growth without addition of auxin in the early passages but may later overcome this need (129).

Cultures of single (separated) cells from the root caps of *Lupinus* have been cultured by Gautheret (130). These increase in size in the presence of a root tip in the medium and are only then stimulated by IAA. Recently Guttenberg (131) has cultured single cells from the stigma slime of *Cymbidium Lowianum* and has obtained large increases in their lengths in the presence of coleoptile tips, which he suggests supply auxin a, and under these conditions further increases are obtained with IAA. Combinations of IAA with thiamine or yeast extract also give some but smaller increases. The evidence is used in further support of his view that IAA merely activates the native auxin. Cell divisions were not obtained. Thus, it has been possible to get elongation, but not yet continued growth of single cells from higher plants *in vitro*.

Growth and normal development of *Datura* embryos and proembryos down to a limiting size of *ca.* 0.20 to 0.15 mm. in length have been obtained *in vitro* on substrates containing mineral nutrients, high sugar content and certain growth factors by Van Overbeek *et al.* (132). Auxin plays a role in this medium at least in determining the form of growth but a more specific embryo factor, originally obtained from coconut milk, is essential at least in the earlier stages. The specific nature of the material is not yet known (133), but Blakeslee *et al.* (134) have found the required material to be present in malt. However, Haagen-Smit *et al.* (135) have found that ten-day-old corn embryos, down to 0.3 mm. in length, will grow on the same medium without the coconut milk fraction and embryos smaller than this stage fail to grow in its presence. The conclusion is reached that growth factors other than those required by *Datura* are needed in the earliest stages of growth of the corn embryo and evidently are supplied from the corn kernels *in vivo*. Anatomical studies of the development of the rye grain by Nutman (136) are pertinent in showing a correlation between separate growth phases of the embryo and embryo sac and the degeneration of certain constituents and adjacent layers. He concludes that substances released during degeneration promote the growth in corresponding phases. [Reference is made to

new reviews of the subject by White (137) and by Larsen (138).]

*In polarity and tropisms.*—In a series of papers Schrank (139 to 142) has studied various agents causing curvatures in the *Avena* coleoptile and finds in each case a transverse electrical polarity arising from the stimulation which precedes and is in the direction to permit the lateral distribution of auxin required for the occurrence of the curvature. The decrease in growth rate in illuminated coleoptiles is preceded by a decrease in potential difference (143). Heitz (144) reports polarity, i.e., germination to occur on the dark side, of unilaterally illuminated moss spores. An extensive treatment of polarity in plants especially in relation to growth and organ formation and effects of auxin in tissue and organ cultures by Gautheret (145) adheres essentially to the concept that polarity is determined by a number of as yet unknown polarly transported hormones specific for organ formation but may have different explanations in different plants. This begs the question as to what causes polar transport. Auxin was the only substance shown on the basis of its morphogenetic effects to be definitely polarly transported. Failure of polar transport of auxin in high concentrations is ascribed to a toxic effect. Nevertheless, papers still appear which imply a nonpolar transport of auxin (146).

The Cholodny-Went theory of tropisms (54, 147) has been further substantiated with respect to the negative and positive geotropism of shoots and roots respectively as a function of the auxin concentration. Geiger-Huber *et al.* (148) show that decreasing the concentration in the root by repeated decapitation tends to make it negatively geotropic (actually ageotropic) and, vice versa, increasing the concentration in the stem by application of IAA to make it positively geotropic. Similarly Kaufmann (149) finds in *Cucumis* hypocotyls that auxin promotes and inhibits growth in the negative and positive geotropic phases respectively. Geotropic stimulation of internodes stimulates release of bound auxin on the lower side (29). Eyster (93) suggests an explanation of hydrotropism based on relations between diastase and auxin. Plagiotropism and apical dominance in *Impatiens Roylei* and some other species have been shown by Snow to be related (150), thus confirming Münch (151). The position of lateral shoots is determined by the balance of the effect of auxin from the terminal bud (or IAA applied to decapitated stems) and effects derived from the leaves

of the lateral shoots. Normally plagiotropic lateral shoots are not inhibited by the terminal bud of the main shoot but on removal of their young leaves they become orthotropic and also subject to inhibition. A further analysis of the torsions of leaves and the effect of applied IAA have been reported by Snow (152).

*In bud inhibition.*—The relation of auxin to bud inhibition is discussed under mechanisms of auxin action. Mitchell (153) has shown that one microgram of 2,4-dichlorophenoxyacetic acid per plant effectively inhibits lateral bud development in beans. Other recent papers are (145, 154, 155). Experiments to test the concept of auxin substrate complex as limiting growth have been carried out with tobacco tissue cultures. The development of buds in submerged cultures was entirely prevented by addition of 0.1 mg. per l. IAA or NAA to the medium. The effect was counteracted by increasing the concentrations of phosphate and sucrose two to four times, when higher concentrations of auxin were required for inhibition. Possibly adenosine, although it inhibits growth by itself may have a similar stimulatory effect when added in combination with auxin. Thus, the substrate concentration seems to determine the growth-promoting versus inhibiting level of auxin.

*In root formation.*—New articles on root formation concern the utilization of new compounds (156 to 161), evaluation of methods (162 to 164) and investigations of factors which in combination with auxin treatments contribute to larger numbers and better growth of roots (165 to 172). Hitchcock *et al.* (162) have compared sixty-three phenoxy compounds with naphthaleneacetic acid on privet cuttings using 24 hr. basal treatments with solutions in the range from 0.32 to 64 mg. per l. They find the activity of 2,4,5-trichlorophenoxyacetic and -propionic acids and 2,4-dibromo- or 2,4-dichlorophenoxy-propionic acids in the range of 2 to 3 mg. to be equivalent to that of 20 to 32 mg. per l. naphthaleneacetic acid. The use of 2,4,5-trichlorophenoxypropionic acid is advantageous because it has little effect on shoot and root modification. The phenoxy compounds with chlorine substitutions in the 2,4,5 or 2,4 positions are more active than those with chlorine substituted singly in either position. Methyl substitution is beneficial in the 2,5 positions but decreases activity when present in other positions.

Van Overbeek *et al.* (171) find that in hibiscus cuttings, sugar

and a variety of nitrogenous compounds in combinations with indolebutyric acid treatments (but not by themselves) effectively replace leaves with respect to the number but not quality of roots formed. Thus, they extend to woody cuttings the results obtained originally with annuals (166). The effect of the leaves is, therefore, ascribed to the general nutrients which they supply. The lack in quality is referred to deficiencies of thiamine and/or other growth factors, which, however, are not calines as specified by Went (173). Nevertheless, much higher concentrations of inorganic nitrogen compounds are needed than of organic. A number of the latter, including urea, which should be adequate sources of nitrogen were ineffective. In the case of ammonium salts there is apparently also an effect of the associated anion. Further studies on the relation of pH of the medium, season of making cuttings and age of material to root formation are reported (167, 172). In the case of tropical species the concentrated solution dip method (175) has been found particularly effective (163, 171). The formation of root hairs as a specific response to auxin was reported by Borgström (176). The effect has also been observed in wheat roots treated with IAA (72). King (177) in a study of the effects of various vitamins and growth substances on *Elodea densa* found that under the conditions of culture only compounds with auxin structure induced the formation of root hairs. In reviewing the literature, Borgström (178) shows that formation of hairs in both roots and stems has been obtained with a variety of agents but only under conditions where they retard elongation and cause thickening. Ethylene, eosin and even sodium chloride have then been effective.

*In flower initiation.*—In many species the change in the apical meristem from vegetative to reproductive growth is characterized by a broadening and thickening of the cells at the expense of elongation. Induction of flowering has been most successfully achieved by manipulation of environmental factors, especially light. Results of this work (179) particularly from translocation experiments (180) indicate the existence of specific reproductive stimuli or flowering hormones. The subject will be discussed here only in relation to effects obtained with auxins and ethylene applications. The first report of a chemically induced change was the reversal from reproductive to vegetative growth in buds of

*Circaea* treated with IAA paste by Dostál *et al.* (181). Conversely Reece *et al.* (182) have shown that in the mango axillary buds are normally vegetative but on removal of the terminal buds and in the presence of leaves, flower formation is induced in the axillary buds. The effectiveness of girdling in other species (183) probably has the same explanation. Chemical induction of flowering has been practiced extensively with pineapple. Its apical meristem must be in a condition of sensitive balance that may be shifted from vegetative to reproductive growth by a number of agents. In fact, the reproductive phase is followed as well as preceded by a vegetative phase in the apex (leading to the formation of the crown) and in some varieties vegetative shoots arise from each fruitlet (184). Injection of ethylene or related unsaturated hydrocarbons into the apex leads to flower induction within 10 days. Auxins are also effective if applied at suitable times (185, 186, 187). Van Overbeek (188) finds treatments with 5 mg./l. solutions (0.25 mg./plant) of naphthaleneacetic or 2,4-dichlorophenoxyacetic acids poured on the apex to be nearly 100 per cent effective in inducing flowering throughout the year in the Cabezona variety grown in Puerto Rico. However, continuous treatment with either ethylene or auxins may delay flower formation indefinitely. Further, it has been shown first by Clark (186) and later by Cooper (187) that flower induction by ethylene may be completely prevented by subsequent application of auxin. The nature of the effect is not known. Van Overbeek (188) has good evidence that the induction is associated with a release of bound auxin from the basal portions of the leaves covering the stem apex, and can be achieved merely by geotropic stimulation. Here again, therefore, the action of the applied chemicals may be primarily on the release of auxin from a bound inactive form. It is of interest that this use of growth substances in field experiments with pineapple also leads to about twenty per cent increase in fruit size (189).

*In parthenocarpy.*—The subject has been reviewed by Gustafson (190). New information concerns the use of new compounds (191, 192) and methods of their application (193 to 197). Zimmerman & Hitchcock (192) find 2-chlorophenoxypropionic-, 2,4-dichlorophenoxyacetic-, 2,5-dichlorobenzoic- and $\beta$-naphthoxyacetic acids to be particularly effective. The activity of a number of halogenated benzoic acids is of special interest in connection

with the earlier suggestion (198) that growth of the fruit is initiated not by the auxin derived from the pollen but by an agent inducing the release of auxin in the tissues.

*In abscission and fruit ripening.*—An auxin effect in preventing abscission of leaves first deduced from observations on the relation of light to leaf fall and proved on *Coleus* by Laibach (199) has been extensively studied by Gardner (200) and successfully applied to the prevention of fruit drop especially in apple and pears. Reference is made to the following papers (1, 6, 201, 202). Faster ripening of immature and freshly harvested fruit (banana, apple and pear varieties) has been obtained by Mitchell *et al.* (203) from treatments with 2,4-dichlorophenoxyacetic acid. Hansen (204) has confirmed the effect on pears and has shown that respiration and ethylene production are increased, but after these processes have been accelerated in mature fruit by cold storage the time of ripening is not hastened significantly. Ethylene and the acid used in combination are more effective than either alone. More rapid maturation and ripening has also been observed from spraying on the trees with naphthaleneacetic acid (205, 206).

*Growth inhibitors, dormancy and rest period.*—Our knowledge of the causes of dormancy, and rest in buds, seeds and tubers is rapidly increasing. Emphasis has shifted from the role of permeability and oxidizing enzymes to specific inhibitory substances as controlling agents. Evenari (207) has summarized the historical development with respect to seed germination and points to the work of Oppenheimer (208) and Köckemann (209) on inhibitors in fruits (blastokolin) as the start of this approach. The work of Ullman (210) and others (211 to 214) indicates that the aglucones of several glucosides are normally occurring effective inhibitors of seed germination and growth. Some of the active compounds are volatile as for example one of the three or more inhibitors present in tomato fruit which has been extensively studied (210, 215, 216). Evidence is accumulating that the substances may be generally respiratory inhibitors some of which affect the sensitivity of response to applied IAA (96, 216, 217, 218). Kuhn *et al.* (104) and Medawar *et al.* (106), have identified some of the active compounds. The work of Nutile (219) on the effect of coumarin on the germination of lettuce seed is of special interest in showing not only that the treatment of the seeds or paper on which they are

placed induces dormancy but reproduces the normal behavior of such dormant seeds with respect to light. A number of studies have shown that inhibitors are present in different layers or tissues of inhibited seeds and that the removal of these parts leads to germination (213, 220, 221, 222). The development may then be abnormal in the early stages as the inhibitors regulate the relative growth of parts of the embryo and young seedling. Opening of the seed coat or partial severance of the inhibiting tissue may be sufficient, but in some cases it must be entirely removed from the medium. The relation of the seed coat and scarification to dormancy may be generally a question of permeability to escaping volatile inhibitors rather than permeability to water. Further work on the maintenance of dormancy or rest period in potato tubers with synthetic growth substances has been reported (223, 224). Denny (225) also has shown a synergistic effect of ethylene chlorohydrin, ethylene chloride and carbon tetrachloride in mixtures of 7:3, 1 (rindite) in breaking dormancy. Thornton (226) has demonstrated the role of oxygen as a controling factor in bud growth and apical dominance in freshly harvested tubers. The use of synthetic growth substances in fall spraying of fruit trees to delay the opening of buds in the spring is further discussed by Hitchcock (227, 228).

*Weed control.*—Tremendous interest has developed in the use of auxin-like compounds, or growth regulators, for weed control. Some of the halogenated phenoxy acids have been found particularly effective. The synthesis and physiological activities of various halogenated benzoic and phenoxy alkyl acids has been reported by Synerholm and Zimmerman (229, 230). Much of the work represents practical testing of effectiveness and selective action of different compounds (191, 231 to 242). Methods of application to the plants and use of carriers has been adapted from work on fruit setting etc. (194, 243, 244, 245). Treatment of the soil before planting may be efficient in suppressing growth of many types of seeds present (246). For more detailed studies mainly 2,4-dichlorophenoxyacetic acid (2,4-D) has been used. Its translocation in the plant (247) and telemorphic effects (248) as well as temperature relations and period of effectiveness (249, 236) have been described. Meristems are preferentially affected, in roots as well as in shoots from aerial applications. After initial

increases both respiration and readily available carbohydrates are markedly reduced (250, 251). Its toxicity to bacteria (252) and in plants fed to animals (253) has also been tested. A possible mechanism for the toxic and selective action of these substances in plants has been discussed in section II. Not merely the halogenated auxin-like acids but substances incorporating the unsaturated lactone structure as well should be tried. A series of eighteen papers containing test methods and trials of herbicidal activity of numerous compounds has been released by the Chemical Warfare Service (254).

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# MARINE BACTERIOLOGY[1]

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The sea contains an extensive microbiological population including bacteria, yeasts, molds, diatoms, dinoflagellates, protozoa, and other microscopic organisms. By virtue of their physiological activities such microorganisms, which vary greatly in diversity of form and in relative abundance, play an important role as biochemical, geological, and hydrobiological agents in aquatic environments. Microbiology is thus a marine science co-ordinate with marine botany, chemical oceanography, marine geology, physical oceanography, and marine zoology (1). Besides being concerned with the effects of microorganisms on the biological productivity of the sea, the transformation of organic matter, and the diagenesis of marine bottom deposits, the marine microbiologist is confronted by practical problems, e.g., problems of sanitary significance, such as fish spoilage (2, 3), shellfish pollution (4), the fouling of ships' bottoms (5), and deterioration of cordage (6), rubber (7), and wooden structures (8) exposed to water. This report is concerned primarily with the biochemical activities of bacteria in the sea.

## ABUNDANCE OF BACTERIA IN THE SEA

*Bacteria in sea water.*—Comprehensive reviews by Benecke (11) and ZoBell (12) indicate that bacteria are widely distributed in the sea, although relatively less abundant than in soil or fresh water. The numbers of bacteria found in sea water, as determined by plating procedures, range from a very few cells to hundreds of thousands of cells per ml. (13). The largest bacterial populations generally occur in water near shore where there is much terrigenous (land-born) pollution (14, 15, 16).

In the open ocean the abundance of bacteria increases from the surface downward to depths of forty or fifty meters and then decreases (17, 18) until, at depths exceeding two hundred meters, there may be fewer than one viable cell per ml. (14). The bacterial population is not detectably restricted by the high hydro-

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static pressure (12), the low temperatures (19) characteristic of the deep sea, nor by solar radiation (20) in surface waters. The paucity of organic matter seems to be the principal factor which limits the bacterial population, a condition which is indicative of the efficiency of bacteria in destroying organic matter in the sea.

*Effect of phytoplankton.*—The abundance of bacteria in sea water appears to be more closely related to the abundance of phytoplankton than to any other ecological factor. Gran (21) observed that the bacteria varied with the diatom content in the Gulf of Maine. A similar parallelism between bacteria and plankton was noted by Waksman *et al.* (22) in sea water, and by Henrici (23) in Lake Alexander.

According to Pütter (24), heterotrophic bacteria in the euphotic zone get much of their food from organic substances secreted by phytoplankton. Although there is some evidence (25, 26, 27) for secretion of organic substances by phytoplankton, their significance in the nutrition of bacteria has been questioned (28, 29, 30). Waksman *et al.* (31) found no evidence that living diatoms nourish saprophytic bacteria, although large bacterial populations were found associated with dead diatoms undergoing decomposition.

*Effect of solid surfaces.*—Bacteria occur only to a very limited extent floating free in the water, most of them being attached to plankton organisms. ZoBell (32) has attributed this to the beneficial effects of solid surfaces which tend to concentrate organic nutrients, retard the diffusion of bacterial enzymes, and provide a resting place for sessile bacteria. The beneficial effects of solid surfaces for bacteria in dilute nutrient solutions have been amply confirmed (33 to 36).

The sessile habit of aquatic bacteria is the basis of the submerged slide technic employed by Henrici (37, 38) and others (39, 40, 41). Kusnetzowa (42) and ZoBell (32) have reported that there are many aquatic bacteria which grow only when attached to solid surfaces. Indirect evidence for the adsorption of organic matter by solid surfaces in sea water has been presented by Harvey (43). Most of the bacteria in marine bottom deposits appear to be adsorbed on or attached to particles of sediment (44).

*Bacteria in bottom deposits.*—It is in bottom deposits where bacteria are most abundant in the sea. Drew (45) found an average of 160,000,000 bacteria per gram of mud from the sea floor near Andros Island in the West Indies as compared with a maximum count of 160 per ml. in the overlying water. Lloyd (46) found

300,000 bacteria per gram of dry mud from the Clyde Sea. Bavendamm (47) detected up to 16,800,000 bacteria per gram (wet weight) of calcareous mud from around the Bahama Islands. Bacterial populations ranging from a few thousand to 500,000,000 per gram have been demonstrated in bottom deposits from inland lakes and seas (48, 49, 50). Mare (51) estimated from plate counts that mud contained from 0.3 to 2 mg. of living bacteria per gram (dry basis).

Wherever profile series have been examined, a progressive decrease in the bacterial population of bottom deposits from the surface downward has been observed (15, 46, 48, 52, 53, 54). Waksman *et al.* (22) found bacteria at the bottom of mud cores 90 cm. long, and Rittenberg (55) detected appreciable numbers at the bottom of mud cores exceeding 350 cm. in length.

*Types of bacteria in marine muds.*—At the mud-water interface aerobes predominate, although most of the bacteria are facultative in their oxygen requirements. With increasing depth of mud, bacteria capable of growing aerobically decrease in abundance more rapidly than those which grow in the absence of free oxygen, and at depths exceeding 10 to 20 cm. anaerobes generally predominate (56). Collectively, the mud microflora is physiologically very versatile, being able to catalyse the transformation of a large variety of organic and inorganic compounds (11, 12, 22, 47, 52, 56 to 59).

## Characteristics of Marine Bacteria

*Morphology.*—The predominant bacteria which Lloyd (46) isolated from Clyde Sea mud were small, gram-negative, asporogenous rods. However, Butkevich (60), who demonstrated from a hundred to a thousand times more bacteria in Barents Sea mud by direct microscopic counts than by plating procedures, concluded that most bacteria in bottom deposits are spore formers. While spore formers (species of the genera Bacillus and Clostridium) may predominate in mud from great depths, the majority of marine bacteria (both species and total numbers) appear to be asporogenous (12). About 80 per cent of the marine bacteria catalogued by ZoBell & Upham (61) are gram-negative rods, and about the same percentage was found to be actively motile by means of flagella. Predominating in the order named were species of Pseudomonas, Vibrio, Flavobacterium, Achromobacter, and Bacterium (12). One or more species of sixty other genera have been found in the sea.

The preponderance of species of Pseudomonas and Vibrio in the sea may be contrasted with the preponderance of species of Bacillus and Actinomyces ordinarily found in soil. There seems therefore to be a characteristic marine bacterial flora (13). Although there are no infallible criteria for the differentiation of marine from nonmarine bacteria, most of the bacteria found in the sea at places remote from possibilities of terrigenous contamination are unlike bacteria from other environments (12, 13, 61).

*Pigmentation and photogenesis.*—Typical of water bacteria, nearly 70 per cent of the bacteria from the sea are chromogenic under favorable conditions (16). Pigmentation is somewhat more common in marine bacteria than in those from fresh-water lakes (62) and far more common than in soil bacteria. Bioluminescence is also preponderantly a property of marine organisms (63), although only a small percentage of the bacteria found in the sea are photogenic. ZoBell (12) has compiled a list of thirty-four species of photogenic bacteria which have been isolated from the sea by various workers.

*Cultural requirements.*—Most marine bacteria require either sea water or isotonic mineral solutions for their growth (64, 65, 66). While there are some euryhaline varieties, the majority of bacteria recently isolated from the sea will not tolerate solutions more hypotonic than twelve atmospheres or more hypertonic than thirty-six atmospheres (12). The osmotic pressure of sea water of average salinity, 3.5 per cent, approximates twenty-four atmospheres. Although marine bacteria are often characterized as halophilic, it is noteworthy that most species are just as sensitive to increased salinity as to hypotonicity. This may be illustrated by the following comparative counts obtained by plating bacteria from various habitats on nutrient media prepared with balanced mineral solutions of different salinities:

| Source of bacteria | Salinity of nutrient media 30% | 15% | 7.0% | 3.5% | 2.0% | 0.5% |
|---|---|---|---|---|---|---|
| | | | average growth index | | | |
| Sea water | 0.0 | 7.4 | 38.3 | **100.0** | 61.7 | 19.2 |
| Marine mud | 0.2 | 6.1 | 43.5 | **100.0** | 54.2 | 23.9 |
| Great Salt Lake | **100.0** | 72.2 | 26.3 | 14.1 | 8.0 | 1.7 |
| Marine saltern | **100.0** | 64.9 | 30.4 | 19.6 | 12.6 | 4.3 |
| Garden soil | 0.4 | 4.6 | 9.8 | 25.2 | 56.1 | **100.0** |
| Sewage | 0.0 | 2.7 | 5.7 | 13.5 | 44.8 | **100.0** |

It is apparent from these data that while marine bacteria are halophilic relative to sewage or soil microflora, they are not nearly so halophilic as bacteria found in marine saltern brines or Great Salt Lake (67). The Russian limans (68), the Dead Sea (69), and other strong brines (70) contain helophiles which grow readily in solutions containing from 20 to 30 per cent sodium chloride, and many of these bacteria actually require 10 to 15 per cent salt for their growth.

*Temperature requirements.*—Since the temperature range of the ocean bottom varies, for the greater part, from 2° to 20°C., it is not surprising that most marine bacteria grow best at relatively low temperatures. Unlike soil or fresh-water microflora, very few marine bacteria grow at temperatures as high as 30°C., and 37°C. may be lethal (71). Although their optimum is generally in the neighborhood of 12° to 25°C., most species of marine bacteria (71, 72) multiply slowly and are otherwise physiologically active at 0° to 4°C. Many species (73 to 76) are physiologically active at −3° to −7.5°C.

## Action of Marine Bacteria on Organic Matter

*Organic content of sea water.*—Organic matter is quite evenly distributed throughout the sea, the content being uniformly small except in the vicinity of land or bottom deposits. An average of 4.2 p.p.m. was found in the open ocean by Krogh (30), who reported further (77) that the ratio of dissolved to particulate organic matter is about 300 to 1. The low concentration of organic matter explains why the sea has been called the world's largest and most efficient septic tank; considerable organic matter enters the sea with terrigenous drainage and more is produced in the sea by photosynthetic organisms but it is quite effectively destroyed.

Much of the dissolved organic matter in sea water is quite refractory, although it gradually undergoes bacterial decomposition when incubated under favorable conditions. Keys, Christensen & Krogh (78) estimated that only 10 to 14 per cent of the dissolved organic matter in sea water was consumed by bacteria, but Waksman & Carey (79) found that from 25 to 50 per cent was decomposed within ten or twelve days at 22°C. ZoBell & Grant (34) reported that after six months' storage in the dark the organic content of sea water was reduced from an initial 4.2 p.p.m. to 0.2 p.p.m. According to Waksman & Renn (80), about 60 per cent of the organic matter decomposed is completely oxidized and

about 40 per cent is converted into bacterial cell substance. The rate of decomposition was faster at 22°C. than at higher or lower temperatures, but after prolonged incubation, as much organic matter was decomposed at 4°C. as at 22°C.

The bacterial oxidation of organic matter in sea water was found by ZoBell (81) to be independent of the partial pressure of oxygen between 0.31 and 12.74 ml. per liter. The proximity to solid surfaces appears to be one of the principal factors which influences the rate at which organic matter in sea water is decomposed (32).

*Decomposition of marine plankton.*—The bacterial decomposition of plankton organisms in sea water is accompanied by oxygen utilization (58, 79, 80), ammonia production, carbon dioxide formation, and phosphate liberation (81, 82). In an experiment conducted by Waksman, Carey & Reuszer (83), about one half of the nitrogen in zooplankton was liberated as ammonia and about one fifth of the carbon as carbon dioxide in nineteen days at 16° to 20°C. In another experiment (84) it was found that the amounts of ammonia produced and phosphate liberated were proportionate to the number of copepods added to sea water.

The bacterial decomposition of mixed net plankton suspended in ten to fifteen liters of sea water and stored in the dark at 20° to 25°C. was observed by von Brand and associates (85). A decrease in the plankton count was accompanied by ammonia production which reached a maximum after fourteen to twenty-five days and then decreased as nitrites and, later, nitrates were produced. Under anaerobic conditions the initial stages of decomposition took place more slowly than under aerobic conditions, and no nitrite or nitrate was developed (86). The nature of the particulate organic matter (87) was of greater importance than the concentration in determining the rate of decomposition. With recurrent supply of plankton, the formation of ammonia, nitrite, and nitrate took place simultaneously (87). Plankton organisms were decomposed at temperatures as low as 1° to 2°C., but the rate of decomposition was more than doubled by an increase of 6° or 8° in temperature (88). Johnson (89) reported the average $Q_{10}$ for marine bacteria to be 2.3 between 5° and 15° and 2.18 between 15° and 25°C. He found that resting bacteria consumed oxygen in sea water at the rate of 2.8 to $185 \times 10^{-12}$ mg. per cell per hour at 25°C.

*Decomposition of carbohydrates.*—Although, as a class, marine

bacteria are somewhat less saccharolytic than either soil or freshwater microflora, virtually all kinds of carbohydrates ranging from simple sugars to complex polysaccharides are attacked by certain marine microorganisms (61). The addition of 4 mg. of glucose per liter of sea water was found by Waksman & Carey (90) to increase bacterial oxygen consumption about threefold, but the glucose was quantitatively oxidized only upon the addition of available nitrogen. Waksman & Renn (80) observed that the rate of glucose oxidation in sea water was directly proportional to the concentration of available nitrogen.

Several species of marine fungi studied by Barghoorn & Linder (91) utilized xylose, galactose, maltose, starch, cellulose, or pectin. Using the minimum dilution method, ZoBell (56) demonstrated 10,000 starch hydrolyzers per gram of marine mud, as compared with 100,000 glucose fermenters and 1000 cellulose digesters. Cellulose-decomposing bacteria were found by Waksman *et al.* (83) to be generally present in sea water and more abundant in bottom deposits and diatom tows. Most of the cellulose decomposers were aerobic, but anaerobic forms were demonstrated. A variety of sugars were utilized by the cellulose decomposers. Bavendamm (47) found aerobic cellulose digesters in all marine mud samples, and anaerobic cellulose digesters in many samples which he examined. Stanier (92) isolated and described four new species of bacteria which digest agar as well as cellulose.

*Agar digestion.*—ZoBell (12) estimated that between 1 and 2 per cent of the colonies which developed on nutrient agar inoculated with sea water or marine mud were agar digesters. Lundestad (93) described seven new species of agar digesters occurring along the coast of Norway. Waksman & Bavendamm (94) demonstrated from 50,000 to 200,000 agar digesters per gram of mud from the Bahama Island region. A new species was isolated which liberated much of the carbon from agar as carbon dioxide. Part of the carbon was utilized for the synthesis of bacterial cell substance. Under unfavorable conditions for growth, the organisms liquefied agar with the accumulation of reducing sugars.

Angst (95) named as new species, thirteen agar digesters from Puget Sound, but unfortunately described them inadequately. Two of the three new species of alginic acid-oxidizing bacteria described by Waksman, Carey & Allen (96) were able to digest agar. Seven species of agar digesters, including five new ones,

were described by Stanier (92). Among the sixty new species of marine bacteria described by ZoBell & Upham (61) seven were agar digesters.

Humm (97) has carefully described all known marine agar digesters, including seventeen new species from the coast of North Carolina, where from 2 to 150 agar digesters were demonstrated per ml. of sea water and from 80,000 to 20,000,000 per gram of mud. All of Humm's agar digesters utilized glucose, fructose, galactose, mannose, cellobiose, and maltose; 90 per cent utilized xylose and lactose; 85 per cent utilized sucrose and salicin; and 53 per cent utilized arabinose, raffinose, and glycerin.

From 10 to 15 per cent of the agar digesters utilized sugars without producing acid and virtually none of them produced gas (97). ZoBell (12) has pointed out that it is a rather common property of marine bacteria to utilize carbohydrates without the production of either acid or gas. This may be attributable to the ease with which organic acids, usually produced during the dissimilation of sugars, are oxidized by marine bacteria. Nearly all of Humm's agar digesters utilized succinic acid, 70 per cent utilized lactic acid, and 65 per cent utilized acetic acid (97). Propionic, citric, malic, gluconic, maleic, malonic, mucic, tartaric, valeric, and butyric acid were also utilized by some cultures. About 70 per cent of the agar digesters attacked alginic acid, 20 per cent attacked cellulose, and 25 per cent attacked chitin.

*Chitin decomposition.*—Carbon dioxide and ammonia production resulted from the decomposition of chitin by marine bacteria studied by ZoBell & Rittenberg (98). Acetic acid and reducing sugars were detected in some cultures. Certain chitinoclastic bacteria utilized purified chitin as a sole source of carbon and nitrogen, whereas others attacked chitin only in the presence of supplementary carbon or nitrogen compounds. Chitinoclastic bacteria appear to be widely distributed in marine bottom deposits (22, 33, 56, 97, 99). Such bacteria have also been found by Hess (100) in large numbers associated with lobsters having a shell disease and on decomposing crabs by Hock (99) and Johnson (101).

After finding chitinoclastic bacteria in eight out of twenty-seven samples of marine salt, Stuart (102) expressed the view that such bacteria may be responsible for damage to skins and hides cured with salt.

***Proteins and other nitrogenous compounds.***—As a class, marine

bacteria are actively proteolytic, rapidly decomposing most nitrogenous compounds with the liberation of ammonia and carbon dioxide. All of the sixty pure cultures studied by ZoBell & Upham (61) liberated ammonia from peptone, forty-seven of them liquefied gelatin, and thirty hydrolyzed casein. Proteolytic organisms predominated in the 530 samples of mud examined by Düggeli (50). Hecht (103) found that proteins were much more susceptible to bacterial decomposition than other components of the bodies of invertebrates, birds, and mammals which he buried in mud. Marine fish are extremely susceptible to bacterial decomposition as manifested by the rapid production of ammonia, indole, trimethylamine, histamine, hydrogen sulfide, and other protein-decomposition products (104 to 107). Plankton organisms, particularly zooplankton rich in proteinaceous material, undergo rapid decomposition in sea water (83, 84).

Asparagine, aspartic acid, glutamic acid, alanine, propionamide, acetamide, sodium hippurate, urea, and creatinine were utilized as a sole source of either nitrogen or carbon by most of the fifteen representative aerobic bacteria of marine origin studied by Ostroff & Henry (108). Cystine, betaine, pyridine, and uric acid were utilized by some of the bacteria. Only one culture utilized tyrosine. Neither guanidine, aniline, nor ethylamine was utilized by any of the organisms (108). From 2 to 4 mg. of glycine, alanine, phenylalanine, glutamic acid, tryosine, and asparagine per liter of sea water were found by Waksman & Renn (80) to be almost quantitatively decomposed by bacteria in two to five days at 20°C. All of the agar digesters studied by Humm (97) could obtain their nitrogen requirements from *l*-leucine, and 94 per cent from either glutamic acid, *d*-arginine, or aspartic acid.

Ammonia is generally liberated from organic nitrogen compounds whenever the nitrogen content exceeds the requirements of the attacking bacteria. Urea is readily ammonified by urea-fermenters, which appear to be quite abundant in the sea (47, 56, 109, 110). Rubentschik (111) found urea-decomposing bacteria in all samples taken from Russian limans. Ammonia was liberated from urea at temperatures as low as −2.5°C.

## Bacterial Transformation of Inorganic Nitrogen

*Ammonia utilization.*—Besides the ammonia liberated by the microbial decomposition of nitrogenous compounds, much am-

monia is excreted by marine animals along with urea, uric acid, etc. Urea is partly dissociated into ammonium and cyanate ions. According to Cooper (112), the equilibrium constant indicates that in sea water urea is mostly dissociated. Appreciable quantities of ammonia also enter the sea in river water and rain (58). Ammonia is assimilated directly and in preference to other forms of nitrogen by many plants (113), including marine phytoplankton (112 to 117). The photochemical oxidation of ammonia in sea water to nitrite and nitrate by sunlight has been demonstrated by ZoBell (118) and confirmed by Rakestraw & Hollaender (119), but owing to the opacity of sea water to ultraviolet radiations, this process is believed to be of no importance below a depth of one meter.

There are no valid estimates on what proportion of the ammonia in the sea is utilized directly by plants as compared with the amount that is oxidized by bacteria to nitrite and subsequently to nitrate. However, data compiled by Rakestraw (120) on the distribution of nitrogen compounds in the sea indicate that relatively large quantities of ammonia must be oxidized by bacteria, particularly below the photosynthetic zone. Nevertheless, conventional enrichment culture procedures generally fail to demonstrate the presence of nitrifying bacteria in sea water except near land (11, 12, 112). Waksman *et al.* (121) concluded that there are few or no nitrifying bacteria in sea water beyond the zone of terrigenous drainage, although active nitrifiers were found in bottom deposits. Similar observations were made by Issatchenko (122) and also by Carey (123), who has reviewed the literature on the subject. The oxidation of ammonia liberated from decomposing plankton in sea water was observed by von Brand *et al.* (85 to 88). Nitrification was inhibited by low temperatures characteristic of deep sea bottoms (124).

The activity of pure cultures of nitrifiers isolated from bottom deposits (125) was found to be favored by redox potential values typical of aerated sea water, but conditions are much too reducing in most marine muds (126) to permit nitrification. Cooper (112), who has marshalled convincing evidence in favor of the intermediate formation of hyponitrite during the bacterial oxidation of ammonia to nitrite, concluded from his studies that immediately above the sea bottom, nitrification is of great importance.

*Oxidation of nitrite to nitrate.*—Most attempts to demonstrate bacteria in the sea which oxidize nitrite to nitrate have yielded

negative results, although such organisms have been found in marine bottom deposits (121, 123), and there is ample evidence that such organisms are functional in sea water (86, 120). The oxidation of nitrite to nitrate is inhibited less by low temperatures, 1° to 2°C., than is the oxidation of ammonia to nitrite (124).

*Nitrate reduction and denitrification.*—Dating from 1899 when Brandt (127) advanced his hypothesis that the bacterial destruction of nitrate limited the growth of phytoplankton in tropical seas, numerous workers [see reviews by Benecke (11) and Waksman *et al.* (121)] have demonstrated a great variety of bacteria in the sea which are capable of reducing nitrate and nitrite in nutrient media, but the extent to which conditions in the sea are conducive to such activities is problematical. According to Waksman *et al.* (121), the activities of denitrifiers are so limited under marine conditions as to render them of little importance in limiting the nitrate content of sea water.

Rakestraw (128) expressed the belief that the bacterial reduction of nitrate may on occasion account for nitrite found in the sea at depths ranging from 25 to 150 meters. Braarud & Klem (129) observed the reduction of nitrate in sea water stored in the dark. The addition of 0.2 mg. of ammonium per liter of sea water retarded nitrate reduction, but it was not stopped by the addition of half this quantity of mercuric chloride. Similar observations were made by Kreps (130), who, after observing nitrate depletion in samples which had been passed through a Seitz filter, credited bacterial enzymes in sea water with the ability to reduce nitrate. Cooper (112) concluded that bacterial denitrification in aerated sea water containing adequate organic matter for energy is thermodynamically possible, but even so, nitrate reduction cannot account for all cases of nitrite occurrence in mid-water.

Drew (131) credited marine denitrifiers with the ability to promote the precipitation of calcium carbonate in tropical seas, a problem which has been extensively studied (47). Accumulating evidence [see reviews by ZoBell (12) and Bavendamm (47)] indicates, however, that except in highly localized environments there is insufficient organic matter in the sea to provide for the activities of denitrifiers.

*Nitrogen fixation.*—The occurrence in the sea of an abundant population of nitrogen-fixing Azotobacter and Clostridium species has been reported (11, 47, 121), but the extent to which nitrogen

fixation actually takes place in the sea is yet to be determined. The salinity requirements of the Azotobacter and Clostridium species found associated with marine algae by Issatchenko (122, 132) led him to believe that these nitrogen fixers were specifically adapted to sea water. Korinek (133) found bacteria in the sea resembling Azotobacter morphologically, but he failed to find any which would fix nitrogen. Except for the sporadic presence of nitrogen-fixing bacteria, there is no evidence of nitrogen fixation in the sea, according to von Brand *et al.* (124).

## Regeneration of Phosphate

The rapid liberation of phosphate from marine plankton undergoing bacterial decomposition has been observed (31, 82, 84, 134). Phosphate was liberated from decomposing zooplankton somewhat more rapidly than from phytoplankton (134). Phosphate was not liberated from bacteria-free diatoms undergoing autolysis, but was rapidly liberated from dead bacterial cells [Renn (135)].

Hydrographic data indicated to Seiwell & Seiwell (134) that most organic decomposition and hence phosphate regeneration takes place above the oxygen minimum layer, roughly 600 to 800 meters. This is in agreement with the observations of Rakestraw (120) and Redfield (136) on the distribution of phosphate in the Atlantic. Herbivorous zooplankton feeding on diatoms were found by Gardiner (137) to excrete considerable quantities of phosphate.

## Microbial Transformation of Sulfur Compounds

*Hydrogen sulfide production.*—In bottom deposits from the Clyde Sea, Ellis (138) found from 10,000 to 3,000,000 saprophytic bacteria per gram, nine tenths of which could liberate hydrogen sulfide from albuminous material. ZoBell (56) demonstrated from 10,000 to 1,000,000 such bacteria per gram of mud from the Pacific Ocean. Under anaerobic conditions, organic sulfur is converted almost quantitatively into hydrogen sulfide by mixed cultures of bacteria occurring in marine mud. Aerobes also liberate hydrogen sulfide from organic compounds, but the sulfide may be spontaneously oxidized as fast as it is liberated in oxygenated sea water.

In stagnant water, hydrogen sulfide may accumulate in concentrations sufficiently high to be lethal for the flora and fauna. Such conditions have been observed in the Black Sea (139, 140, 141), the Caspian Sea (49), Odessa limans (140, 143), Lake Ritom

(144), Norwegian fjords (145), brackish water (146), parts of the Atlantic Ocean (147), and in other bodies of water having poor vertical circulation. The hydrogen sulfide has been shown to result from the bacterial decomposition of organic matter and, more important, from the reduction of sulfate.

*Sulfate reduction.*—The occurrence of sulfate-reducing bacteria in marine environments has been recorded by several investigators (47, 56, 109, 147 to 150). Rittenberg (151) found such bacteria in numerous mud samples from the Pacific Ocean, but only rarely were sulfate reducers detected in sea water from the open ocean. A decrease in the sulfate content of mud with increasing core depth is indicative of the activity of sulfate reducers *in situ*. The low concentration of sulfate in many oil field waters together with the presence of sulfide (152, 153) is also suggestive of the action of sulfate reducers in ancient marine sediments. The reported presence of sulfate-reducing bacteria in oil-well brines (154, 155, 156) would be more significant if shown to be indigenous species and not merely adventitious organisms introduced by drilling or pumping operations. The demonstrated ability of sulfate reducers to modify hydrocarbons (148, 157 to 160) is noteworthy, although the part played by bacteria in the formation and transformation of petroleum is still indeterminate.

From North Sea coast water, van Delden (161) isolated a sulfate reducer, now known as Desulfovibrio aestuarii, which differed from other varieties primarily in that it required sea water or 3 per cent sodium chloride for its growth. Baars (162), however, claimed to have acclimatized the marine organism to grow in fresh-water medium, an observation which Rittenberg (151) was unable to repeat with sulfate reducers isolated from the sea. According to Rittenberg (151), the maximum rate of hydrogen sulfide production in 3 per cent salt medium was about $8.4 \times 10^{-11}$ mg. per cell per hour at 30°C. The $Q_{10}$ for sulfate reduction in 3 per cent salt medium was 2.3 between 20° and 30°C. The marine sulfate reducer used neither nitrate, phosphate, selenate, tellurate, sulfonate, sulfone, nor sulfoxide as hydrogen acceptor.

*Sulfur bacteria.*—At least twenty species representing seven genera of achromic sulfur bacteria, which oxidize sulfide, elementary sulfur, thiosulfate, or tetrathionate, have been found in the sea (12). Some of these appear to be exclusively marine species (138, 143), while others live in both marine and nonmarine habitats

(47, 138, 163, 164). Achromic sulfur bacteria, along with purple forms (138, 163, 164, 165), are abundant in shallow water where hydrogen sulfide is being produced. Whether the hydrogen sulfide is oxidized to free sulfur or to sulfate depends upon the bacterial species, oxygen tension, concentration of hydrogen sulfide, light penetration, and other factors (166, 167).

In localized regions, purple bacteria sometimes occur in sufficient abundance to impart a red or "bloody" coloration to the water (138, 165, 168). They are independent of salinity throughout the range of 0.05 to 7.5 per cent sodium chloride, according to Baas Becking (164), who states further that they prefer diffuse or subdued sunlight, a low oxygen tension, and hydrogen sulfide, which is provided largely by sulfate reducers. Further information on the physiology of this group of bacteria is given in treatises by van Niel (166, 167, 169) and Gietzen (165), the latter dealing specifically with marine Thiorhodaceae.

## Activities of Marine Microorganisms as Geological Agents

*Calcium carbonate precipitation.*—Several investigators (45, 47, 131, 170, 171, 172) have demonstrated the ability of certain bacteria to promote the precipitation of calcium carbonate in sea water enriched with nutrients. Lipman (173, 174) questioned the ability of bacteria to precipitate calcium carbonate from sea water to which no nutrients were added, a conclusion which was based upon the paucity of bacteria found in the open ocean. However, this objection has been nullified by the observed abundance of bacteria in calcareous bottom deposits (47).

Either denitrification (45, 131), sulfate reduction (170), ammonia production (172), oxidation of organic acids (175), or other microbiological processes which increase the pH may promote the precipitation of calcium carbonate. The reverse processes, which take place in certain parts of the ocean, tend to dissolve calcareous deposits (12, 47). Bavendamm (47), who has summarized the literature on the subject, concluded that calcium precipitation in tropical seas is primarily a microbiological process, but that there are no specific "calcium bacteria" endowed with this ability. Such processes take place only in localized sections of the ocean. In his review of the literature, Baier (175) claimed that microorganisms may influence calcium carbonate equilibrium by their effects on

the pH, by producing or consuming carbon dioxide, by oxidizing organic calcium salts, or by assimilating calcium.

*Deposition of iron and manganese.*—There are several ways in which bacteria are instrumental in the deposition and transformation of iron and manganese (176, 177, 178). The development of iron bacteria in saline chalybeate waters has been demonstrated by Cholodny (179). Thiel (180) reported the occurrence of numerous heterotrophic bacteria which precipitated iron and manganese in marine muds. Species of iron-depositing Gallionella were isolated from the Petschora Sea and White Sea by Butkevich (181). Bacteria were believed by Issatchenko (182) to be the chief agents in the formation of pyrite.

*Effects of bacteria on pH and $E_h$ of sediments.*—Bacteria appear to be the principal dynamic agencies which influence the hydrogen ion concentration and oxidation-reduction potential of bottom deposits (126, 183), by catalyzing the transformation of both organic and inorganic constituents of mud. In the presence of organic matter, bacteria create reducing conditions by consuming oxygen and producing hydrogen sulfide and other reducing agents. The oxidation-reduction potential as well as the hydrogen ion concentration is believed to have a pronounced effect on the composition, chemical reactivity, diagenesis, color, biological population, and other properties of recent sediments (126).

The ability of bacteria to consume oxygen in bottom deposits (53, 81, 184, 185) more rapidly than it can be replaced by diffusion, oceanic circulation, or photosynthetic processes promotes reducing conditions. In a reducing environment aquatic bacteria tend to attack organic matter with the formation of organic complexes, methane, hydrogen, hydrogen sulfide, carbon dioxide, ammonia, and phosphate, whereas under aerobic or oxidizing conditions the last three named products together with sulfate predominate. Reducing conditions in marine sediments are believed to favor the conversion of organic matter into petroleum hydrocarbons (186).

*Role played by bacteria in petroleum formation.*—By splitting nitrogen, sulfur, phosphorus, and oxygen from organic matter, marine anaerobes tend to increase the percentage composition of hydrogen and carbon, thereby producing substances which are more petroleum-like than the original organic matter (187). Methane was the only hydrocarbon observed by Thayer (188) to

result from the anaerobic fermentation of either diatoms or fatty acids mixed in marine muds, but a liquid oil resembling balkhashite was obtained by Sturm & Orlova (189) from the action of anaerobes on palmitic acid and other lipids. According to Stadnikow (190), lipolytic anaerobes growing on fat-rich algae produced unsaponifiable compounds, including some petroleum hydrocarbons. A slight increase in the unsaponifiable content of decomposing algal lipids was observed by Rosenfeld (191). In similar experiments reported by Clarke & Mazur (192), diatoms stored with mud showed a decrease in the amount of free fatty acids and an increase in hydrocarbons. While methane is the only hydrocarbon observed to be produced in large quantities by bacteria, small amounts of paraffin hydrocarbons in the range of lubricating oil and wax have been shown by infrared and ultraviolet absorption studies to be produced by certain sulfate-reducing bacteria (193).

Ginsburg-Karagitscheva (194) regards it as established that petroliferous formations have been inhabited by active microflora. He reported that anaerobic bacteria from the Black Sea transformed albuminous materials and fatty substances by processes of hydrogenation, decarboxylation, and polymerization into oil-like products having a bituminous odor. Phenol and *p*-cresol, rather common constituents of crude oils, are produced by the action of bacteria on *l*-tyrosine (195). The production of benzene and benzoic acid from tyrosine and phenylalanine has been reported in the earlier literature, but this observation has not been amply confirmed. While there is incontrovertible evidence for the bacterial production of hydrocarbons besides methane and certain carotenoid pigments (196), there are no reasons for believing that bacteria have produced petroleum, although bacterial activity may have contributed to petroleum genesis in various ways, particularly by conditioning organic matter in marine sediments.

*Bacterial oxidation of hydrocarbons.*—The presence in marine sediments of bacteria which are endowed with the ability of attacking petroleum hydrocarbons (197) suggests that such bacteria may prevent the accumulation of oil in certain environments. From water, marine sediments, and oil-soaked soil nearly a hundred species of microorganisms representing thirty genera have been described which attack one or more types of hydrocarbons (160). Most of these organisms function only in the presence of free oxygen, but recently it has been demonstrated that hydro-

carbons are attacked by anaerobic sulfate reducers (148, 158, 198). Such bacteria may have played an important role in the modification of petroleum in certain deposits.

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# ANTIMALARIAL DRUGS

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Following the attack on Pearl Harbor and the subsequent fall of Java, from which ninety per cent of the pre-war supply of quinine was obtained, the necessity of conducting extensive military operations in some of the most highly malarious regions of the world evolved a coordinated attack upon the malaria problem on a scale never before contemplated. In consequence, a tremendous amount of information on both old and new antimalarial drugs has been accumulated. An adequate summary of this information is not possible at present because the publication of the results of many important investigations has been impeded by war time restrictions. A good deal of this information, including the results of the assay for antimalarial activity of more than 14,000 substances conducted under the auspices of the Committee on Medical Research of the Office of Scientific Research and Development, will appear in a monograph entitled *A Survey of Antimalarial Drugs* (1), in press at the time of this writing. This monograph contains adequate descriptions of all techniques utilized in this country for the evaluation of potential antimalarial drugs in both animals and man, the results of the pharmacological examination of many of these drugs in animals, and the results of controlled clinical trials in man of approximately one hundred antimalarial drugs. Little of this information has yet been published elsewhere. Reviews of the older literature on antimalarial drugs (2 to 8), and summaries (9, 10) of hitherto unpublished German investigations of such substances are available. Accordingly, the contents of this review are limited to a summary of the principal developments in the study of antimalarial drugs which have taken place since 1940.

## QUINACRINE

This valuable synthetic drug was introduced in Germany under the name of atebrin, in 1932 (11), although not until the following year (12) was its structure publicly disclosed to be 6-chloro-9-(4-diethylamino-1-methylbutylamino)-2-methoxyacridine. Numerous

related derivatives of acridine exhibit antimalarial activity (1, 13), but as yet none of these appears to have been given an adequate clinical trial in man.

Prior to 1942, quinacrine had been extensively used for the treatment of individuals suffering from malaria, although no exact information concerning the pharmacological behavior, efficacy, limitations of usefulness, or proper dosage schedules of this drug was available at the outset of the war in the Pacific (14, 15). Yet this drug, subsequently manufactured on an enormous scale in this country (16) and in England, played a major role in determining the success of military operations in tropical climates (17). When administered regularly in appropriate dosage, quinacrine prevents the development of parasitemia and frank clinical symptoms in individuals who become infected with malaria as a result of being bitten by infected anopheline mosquitoes.

Quinacrine is not a causal prophylactic; that is, it does not destroy pre-erythrocytic forms of the various species of *Plasmodium*. The usefulness of this drug stems from its ability to inhibit the intra-erythrocytic reproduction (schizogenous cycle) of the malaria parasite, which, in the absence of an antimalarial drug, is responsible for the characteristic symptoms of the malarias. Overt attacks of malaria are quickly aborted by proper treatment of the afflicted individuals with quinacrine. If such attacks are due to *P. falciparum*, a radical cure of the infection results from a proper course of quinacrine therapy. Such is not the case in *P. vivax* infections. While overt attacks of naturally induced vivax malaria are readily abolished by the proper use of quinacrine, a radical cure of this infection is not achieved. This is shown by the fact that some weeks after the last dose of quinacrine a relatively large proportion of treated patients suffer relapse (18, 19). This tendency to relapse is characteristic of vivax malaria, and it is a major problem of chemotherapy to find a drug which will effect a radical cure of this type of the disease.

As with other chemotherapeutic agents, the successful use of quinacrine for control of the human malarias is predicated upon adherence to appropriate dosage regimens. The establishment of the proper dosage regimen of quinacrine for the prompt termination of overt attacks of malaria is largely the outcome of comprehensive investigations (20, 21) of the physiological disposition of

this drug following different dosage regimens in animals, in normal humans, and in patients. These investigations disclosed that quinacrine is readily absorbed from the gastrointestinal tract, that a relatively small fraction of the daily dose is excreted in the urine, and that this drug is extensively localized in the tissues. In the liver and spleen, for example, such localization may result in a concentration of quinacrine 10,000 to 20,000 times that of the plasma.

Experiments on patients with induced malaria disclosed that the minimum concentration of quinacrine in the plasma required for prompt alleviation of symptoms and of parasitemia is 30 μg. per l. in vivax malaria and 50 μg. per l. in falciparum malaria when maintained respectively for periods of four and of six days. While the plasma concentration of quinacrine attained in different individuals on the same dosage regimen of this drug is subject to marked variation, the results of this investigation allowed the selection of an oral dosage of quinacrine sufficiently high to maintain plasma concentrations in excess of the critical levels just mentioned. Once this had been established, it quickly became apparent that the antimalarial efficacy of quinacrine is much greater than had been supposed prior to 1942. This has been substantiated by reports (22, 23, 24) of experience with naturally induced malaria acquired in the field. Indeed, it is probably safe to conclude that when used in appropriate dosage quinacrine is superior to quinine in every respect.

The significance of the plasma concentration of quinacrine in determining the effective use of this drug has recently been questioned as a result of the observation that in ducks infected with *P. lophurae*, there is little correlation between the suppression of parasitemia and the concentration of quinacrine attained in the plasma. In this instance, the efficacy of this drug appears to be determined by the magnitude of the oral dosage (25).

Investigations of the type just considered cannot be conducted without recourse to methods for the determination of quinacrine in tissues and body fluids. Several such methods have been described (26 to 29), but only one of these (27) appears to be sufficiently specific to differentiate between quinacrine and its fluorescent metabolic products, which may occur in blood, and certainly occur in urine (see below). Ammonium oxalate must not be used as an anticoagulant for the preparation of plasma intended for

analysis, since this salt displaces quinacrine from leukocytes, thereby giving rise to fictitiously high plasma concentrations (30).

Simple, albeit nonspecific, methods for the determination of the approximate quinacrine concentration in urine have been proposed (31 to 34). The observation that the quinacrine clearance from plasma is directly proportional to the rate of ammonia excretion (35, 36) is the basis of a field method for the estimation of plasma quinacrine concentrations from the results of urine analyses (37, 38).

Indirect evidence for the existence of metabolic degradation products of quinacrine in the blood and urine of recipients of this drug has been obtained by analytical procedures involving differences in solubilities (27, 39, 40). Such metabolic products have been isolated from the urine of dogs following ingestion of quinacrine (41), although the quantities obtained were insufficient for the precise identification of their chemical nature. From the urine of humans treated with the commercially available *dl*-form of quinacrine, only the *l*-isomer can be isolated (42). This is accompanied by three metabolic products derived from quinacrine, believed to be identical with those excreted by dogs. The three metabolic degradation products of quinacrine isolated from the urine of humans have been identified (43, 44) as 6-chloro-9-(4-diethylamino-1-methylbutylamino)-2-hydroxyacridine, 9-amino-6-chloro-2-methoxyacridine, and 9-amino-6-chloro-2-hydroxyacridine, by polarographic comparison with authentic samples of these substances.

## Derivatives of 4-Aminoquinoline

The attachment of a dialkylaminoalkylamino side chain to position 4 of the quinoline nucleus results in the formation of compounds which exhibit antimalarial activity. This activity is markedly enhanced by the introduction of appropriate substituents into positions 6 or 7 of the quinoline nucleus. Substances of this type are described in patents issued to German investigators (45, 46, 47), and in the Russian literature (48). Although some representatives of this class of substance were known to exert a quinine-like action in parasitized birds (49, 50), until recently little or no attempt was made to exploit their potentialities for the treatment of the human malarias. Unlike the isomeric derivatives of 8-aminoquinoline, derivatives of 4-aminoquinoline do not

exhibit gametocidal activity (49). Like the derivatives of 9-aminoacridine, their antimalarial activity appears to be limited to an inhibition of the asexual multiplication of intraerythrocytic forms of various species of *Plasmodium*. This is not surprising, since acridine is a 2,3-benzoquinoline, and position 9 of the acridine nucleus is comparable to position 4 of the quinoline nucleus.

Although a great many derivatives of 4-aminoquinoline have been prepared and assayed for antimalarial activity during the past few years (1), only a few of these substances can be mentioned here.

Because of its reported quinine-like action in parasitized birds (49), 4-(4-diethylamino-1-methylbutylamino)-6-methoxyquinoline has been prepared (51) in quantity sufficient for an evaluation of its antimalarial potency in man. This evaluation has shown this substance to be practically equivalent to quinine, but inferior to quinacrine in the therapy of the human malarias (1).

Two similar compounds first prepared in Germany (9, 46, 47) have been found to be more potent, in both avian and human infections, than quinine or any of the known derivatives of 4-amino-6-methoxyquinoline. These are SN 6911, 7-chloro-4-(4-diethylamino-1-methylbutylamino)-3-methylquinoline; and SN 7618, 7-chloro-4-(4-diethylamino-1-methylbutylamino)-quinoline. The antimalarial activity of SN 6911 is substantially equivalent in both its qualitative and quantitative aspects to that of quinacrine (1). Unlike the latter, however, it is colorless, and consequently its use in man is not attended by a discoloration of the skin, which some users of quinacrine find highly objectionable. Although the synthesis of SN 6911 presents some difficulties, it is likely that it might have come to enjoy extended use, had not SN 7618 proved to be more efficacious in both the suppression and therapy of the human malarias. Because of this, the synthesis of the latter drug has been reinvestigated (52), and important improvements have been made in the synthesis of both SN 7618 (53) and of 4,7-dichloroquinoline (54), an important intermediate in its preparation. Under the official name of chloroquine, SN 7618 has been introduced into medicine (55).

In man, the general behavior of chloroquine parallels that of quinacrine. Like the latter drug, chloroquine is extensively degraded in such a fashion that only ten to twenty per cent of the

daily dose is excreted in the urine. The nature of the metabolic degradation products is under investigation (56). In both avian and human infections, chloroquine is decidedly more potent than quinacrine. Although this increase in potency permits a rapid termination of acute attacks of both falciparum and vivax malaria, and a radical cure of the former disease, it does not affect the tendency towards relapse of individuals infected with *P. vivax*. The greater antimalarial potency of chloroquine relative to that of quinacrine is especially evident in its efficacy in suppressing the development of parasitemia, and the onset of overt attacks of malaria in infected individuals. For this purpose, a single dose of 0.3 gm. of chloroquine taken on the same day each week is adequate (55).

## Derivatives of 8-Aminoquinoline

Since the introduction of pamaquine (plasmochin) [8-(4-diethylamino-1-methylbutylamino)-6-methoxyquinoline], a tremendous number of derivatives of 8-amino-6-methoxyquinoline have been examined for antimalarial activity. Only one of these derivatives is of sufficient interest to warrant comment here. This is SN 13,276: 6-methoxy-8-(5′-isopropylaminoamylamino)-quinoline (57). This new drug, designated pentaquine, is definitely less toxic than pamaquine. Administered in conjunction with quinine to patients suffering from a standardized experimental infection of vivax malaria, it effects a radical cure of the disease. Similar treatment of Army personnel suffering from naturally acquired infections of this type of malaria has resulted in a precipitous drop in the relapse rate (58). Neither of these highly desired results has been attained with any drug other than pamaquine and pentaquine. The greater toxicity of the former has precluded its general acceptance for this purpose.

By use of a newly developed technique of analysis involving an ingenious application of countercurrent extractions (59, 60), commercial samples of pamaquine have been found to be badly contaminated with an isomeric entity resulting from an unexpected rearrangement of the side chain of this drug during the course of synthesis. As a result of this observation, pure pamaquine has been prepared for the first time (61). Interestingly enough, as determined in animals neither the toxicity nor the antimalarial potency of this preparation differs significantly from those of the commercially available product (62).

## QUININE

Animal tissues contain an enzyme capable of catalyzing the oxidation of quinine (63), other cinchona alkaloids (64), quinoline, and some of its derivatives, and N-methylnicotinamide (65). The product resulting from the oxidation of quinine through the intervention of this enzyme, derived from rabbit liver, has been isolated (66) and identified (67) as a 2′-hydroxy derivative of quinine, 1-2′-hydroxy-6′-methoxy-3-vinyl-ruban-9-ol (for nomenclature see 68). The 6′-desmethoxy analogue of this substance, as well as a more highly oxidized derivative, has been isolated from human urine following ingestion of cinchonine (64). Oxidation products of analogous nature have been isolated from human urine following ingestion of quinidine and cinchonidine (64). The precise structure of these substances has not yet been determined.

Impure quinine oxidase has been isolated from rabbit-liver and found to contain riboflavin. This enzyme is associated with aldehyde oxidase. Anaerobically it is reduced by cinchonine, while under aerobic conditions hydrogen peroxide is formed as the oxidation of the substrate proceeds. Interestingly enough, in the presence of this enzyme and of 2′-hydroxyquinine, the anaerobic oxidation of an aldehyde is accompanied by a reduction of the former to quinine (65).

In chickens infected with *P. lophurae* and with *P. gallinaceum* respectively, 2′-hydroxyquinine is reported to be only 0.05 to 0.25 as potent as quinine (69). Thus it appears unlikely that the antimalarial action of quinine is predicated upon its conversion to this substance.

Analytical methods for the determination of quinine in body fluids and tissues have been devised (70, 71, 72). One of these methods (71) is adapted to the determination of other organic bases as well, and by combination with a suitable extraction procedure may be used to estimate both the concentration of the cinchona alkaloids and their metabolic degradation products in the same sample.

By the use of this method it has been possible to establish the critical plasma concentration of quinine necessary for the clinical cure of trophozoite induced vivax malaria (McCoy) as between 4 and 5 mg. per l. (73). This is an important reference standard for evaluation of the relative potency of other antimalarial drugs. In the same infection, the critical plasma concentrations of other cinchona alkaloids have been found to be: for

cinchonidine, 2 mg. per l.; for quinidine, 0.9 mg. per l.; for cinchonine, less than 0.1 mg. per l. (74). Differences in the ease with which these related alkaloids suffer metabolic degradation is assumed to be a major factor in the determination of their concentration in plasma (75). Evidence for this is derived from a detailed study of the metabolic fate of cinchonine. Less than 5 per cent of the oral dose of this substance can be recovered from human urine, which also contains a quantity of 2′-hydroxycinchonine and of a more highly oxygenated derivative, equivalent respectively to 55 per cent and 25 per cent of the parent alkaloid administered. The renal clearance of 2′-hydroxycinchonine approximates that of the renal plasma flow; yet, despite this prompt elimination, a portion of this substance suffers further oxidation (76).

### 4-Quinolinemethanols

Consideration of the nature of the metabolic products of the cinchona alkaloids resulted in the suggestion that the potency of these drugs might be enhanced by the introduction of substituents into the 2′-position of their component quinoline nuclei, thus blocking the point of attack of quinine oxidase. By the interaction of cinchonine and phenyllithium, 2′-phenyldihydrocinchonine was obtained (77). This substance proved to be definitely more potent than the parent alkaloid when assayed in parasitized birds. Because of technical difficulties involved in the preparation of compounds of this type, attention was turned to analogous derivatives of 4-quinolinemethanols.

In general, two classes of these substances have been extensively investigated. Representatives of these two types are 6-methoxy-α-2-piperidyl-4-quinolinemethanol (78), and 6-methoxy-α-dihexylamino-4-quinoline methanol (79). The introduction of a phenyl group into position 2 of the quinoline nucleus of such compounds has been found to result in a definite enhancement of antimalarial activity. Numerous substances of this type, i.e., derivatives of 2-phenyl-4-quinoline methanol, have been prepared (80 to 83), and many of these have exhibited an antimalarial potency markedly greater than that of quinine (1). Space does not permit further discussion of substances of this class, other than to note that in both types of compound derived from 4-quinoline methanol the incorporation of other substituents into the aromatic nucleus frequently results in a marked elevation of potency, as determined by conventional assays in parasitized birds. Thus SN

10,275, or 6,8-dichloro-2-phenyl-α-2-piperidyl-4-quinolinemethanol appears twenty and eighty times as potent as quinine when assayed in ducks infected respectively with *P. lophurae* and *P. cathemerium* (84). In man infected with *P. vivax*, this substance is no more potent than is quinine (84).

In passing, it may be noted that discrepancies of this sort between the results of avian assays and clinical trial in man are not uncommon. This and related difficulties in the evaluation of the potentialities of antimalarial drugs have recently been discussed for the first time (84).

That the quinoline nucleus is not necessary for exhibition of antimalarial activity is evident from the fact that the introduction of a dialkylaminomethylene group into the α-position of substituted methanols derived from cyclic hydrocarbons frequently results in the formation of substances whose antimalarial potency compares favorably with that of quinine. Many substances of this type have been prepared (1, 85). Two representative compounds are SN 1796, α-diamylaminomethyl-1,2,3,4-tetrahydrophenanthrene-9-methanol; and SN 5241, α-dinonylaminomethyl-1,2,3,4-tetrahydrophenanthrene-9-methanol. Both these substances exhibit a potency equivalent to one fourth that of quinine in chicks infected with *P. gallinaceum*, and appear to be equivalent to quinine in the therapy of human malaria (85), although toxic side reactions have restricted their use in patients (1).

## Derivatives of Pyrimidine

Numerous derivatives of pyrimidine have been prepared (86 to 92), in the expectation that substances of this class would exhibit an antimalarial activity comparable to that of sulfadiazine. In this series of compounds, antimalarial activity results when both an arylamino group and an alkylaminoalkyl group are attached to the pyrimidine nucleus in a fashion which permits of prototropic change (93). Such a change is facilitated by the inclusion of a guanidine moety between the aromatic and pyrimidine nuclei. Of several compounds so constituted, one, M 3349, 2-*p*-chlorophenylguanido-4-(2-diethylaminoethylamino)-6-methylpyrinidine, was found to be a fairly potent antimalarial when assayed in parasitized birds (93). In man this drug effectively suppresses the three types of human malaria (94, 95) but it is less potent than quinacrine and chloroquine.

Theoretical speculations (96) concerning the mode of action of

M 3349 and related derivatives of pyrimidine indicated that in such compounds a portion of the pyrimidine nucleus could be discarded without loss of antimalarial activity. This directed attention to an examination of the potentialities of derivatives of biguanide and thus opened an entirely new chapter in the history of antimalarial drugs.

## Derivatives of Biguanide

Several $N^5$-diaklyl derivatives of 1-*p*-chlorophenylbiguanide were found to exhibit antimalarial activity. The most potent member of this series of compounds is M 4430, $N^1$-*p*-chlorophenyl-$N^5$-isopropyl biguanide. In parasitized birds, the antimalarial activity of this substance resembles that of sulfadiazine (see below). Like the latter drug, M 4430 fails to suppress the development of the intraerythrocytic forms of *P. cathemerium;* it will, however, inhibit the development of these forms of *P. gallinaceum* and is a causal prophylactic against sporozoite infection or chicks with this species of parasite (96). In man, M 4430 appears to be a partial prophylactic against the New Guinea strains of *P. vivax* (97), and its schizonticidal action appears equal to that of quinine both in vivax malaria (97, 98) and falciparum malaria (98). Severe symptoms of toxicity have been observed in patients treated with large doses of this drug.

The most important member of the biguanide series of drugs is M 4888, $N^1$-*p*-chlorophenyl-$N^5$-isopropylbiguanide acetate, now marketed under the name of paludrine, a name originally applied to the above mentioned pyrimidine derivative, M 3349 (99). Unlike M 4430, $N^1$-*p*-chlorophenyl-$N^5$-methyl-$N^5$-isopropylbiguanide, paludrine inhibits the development of the intraerythrocytic forms of all species of the malaria parasite so far examined in birds (96), and those of *P. vivax* and *P. falciparum* in man (100, 101, 102). A comprehensive examination of all aspects of the use of paludrine for the control of human malarias has led to the conclusion that this drug is definitely superior to all other known antimalarial drugs (102). Paludrine is a causal prophylactic against mosquito-induced falciparum malaria, and a partial prophylactic against mosquito-induced vivax malaria; it is a potent schizonticide, doses of 100 mg. twice weekly suppressing effectively the development of overt attacks in individuals infected with the causative parasites of these infections. Radical cures of falciparum

malaria are readily achieved by the administration of appropriate doses of paludrine, whereas similar cures of vivax malaria are not a consequence of treatment with this drug. Such cures, however, have been obtained by the conjoint administration of paludrine and pamaquine. As gauged by the relapse rate in patients so treated, this combination of drugs is as effective as that of quinine and pamaquine, now in use. In those patients with vivax malaria who suffer relapse, the period between relapses following treatment with paludrine and pamaquine is approximately twice as long as in the case of patients treated with quinine and pamaquine (102).

Paludrine does not possess gametocidal properties, but, if it is present in human blood containing gametocytes of either *P. vivax* or *P. falciparum* at the time of ingestion by mosquitoes, the sexual development of these parasites in the insect host is inhibited so that sporozoites are not formed. In the case of *P. falciparum* this inhibition occurs prior to the formation of oöcysts; in the case of *P. vivax*, oöcysts develop, but subsequently die (102). This fact is of great sanitary significance, for this unique action of Paludrine should materially decrease the chances of infection in areas where the drug may find extensive use as a suppressant.

Besides possessing the unique properties noted above, Paludrine is probably the least toxic antimalarial drug known; owing to its simple constitution it also can probably be prepared more easily and inexpensively than any other synthetic antimalarial drug. These are important considerations in the case of any drug intended for large scale use.

## Sulfonamides and Metanilamides

The schizogenous cycles of some species of *Plasmodium* is readily inhibited by sulfonamides. In monkeys, *P. knowlesi* infections are suppressed by sulfanilamide (103), and by sulfadiazine (104). Eradication of this infection has been achieved with the former drug. Sulfanilamide is without effect on the course of *P. inui* infections in monkeys (103). This difference in susceptibility to sulfonamides is exhibited by other species of *Plasmodium*. Thus, drugs of this class are relatively ineffective supressants of *P. cathemerium* infections in ducks (105). Treatment with sulfadiazine eradicates the exoerythrocytic forms of *P. gallinaceum* in chicks (106, 107, 108), but is without effect upon the corresponding forms of *P. elongatum* in canaries (109).

Sulfadiazine is a causal prophylactic against mosquito-borne infection with *P. gallinaceum* in chicks (107, 108). A similar prophylactic action of proseptasine ($N^4$-benzylsulfanilamide) in man has been claimed (110), but seems doubtful in view of the observation that in man sulfadiazine, sulfamerazine and sulfamezathine are not prophylactic against either *P. vivax* or *P. falciparum* (111). Each of these drugs in doses of 1 gm. per day will suppress, and in some cases cure, falciparum malaria, but fail to prevent the onset of overt attacks of vivax malaria in the majority of infected individuals (111).

The simultaneous administration of *p*-aminobenzoic acid and a potent sulfonamide, such as sulfadiazine, to birds infected with species of *Plasmodium* susceptible to drugs of this type allows the infection to proceed unabated (112, 113, 114). This is not true in the case of certain halogenated sulfonamides, such as 3′,5′-dibromosulfanilanilid and its 3′,5′-dichloro analogue, whose antimalarial activity is not completely antagonized by *p*-aminobenzoic acid. Unlike the better known sulfonamides, these halogenated derivatives are effective suppressants of *P. cathemerium* infections (105); hence the mechanism of their antimalarial action must differ from that of sulfadiazine and related compounds.

A number of derivatives of metanilic acid has been prepared (115) in the hope that they might prove useful in the therapy of human malarias. Assays of these derivatives in chicks infected with *P. gallinaceum* have shown several substances of this type to be more potent than quinine or sulfadiazine. For example, in this infection, 2-metanilamido-5-chloropyrimidine (metachloridine) and its bromo and iodo analogues are at least sixteen times as potent as sulfadiazine. Like the latter, these new derivatives of metanilic acid are prophylactic against sporozoite (*P. gallinaceum*) inoculation, but they are incapable of eradicating an established infection. Unlike sulfadiazine, metachloridine effectively suppresses *P. cathemerium* infections (116).

## MISCELLANEOUS DRUGS

*α-Aminocresols.*—Many derivatives of α-amino-*o*-cresol formed by the condensation of phenols with formaldehyde and dialkylamines (Mannich reaction) have been found to be effective suppressants of avian malarias (117). For example, assays in chicks infected with *P. gallinaceum* have shown both SN 8316, 6,6′-

dialkyl-$\alpha,\alpha$-*bis*-dimethylamino-4,4′-bi-o-cresol, and SN 10,271, $\alpha,\alpha$-*bis*-diethylamino-5,5′-bi-o-cresol to be four times as potent as quinine.

*Antibiotics.*—Relatively large doses of penicillin do not influence the course of therapeutically induced vivax malaria in man (118). In chicks infected with *P. gallinaceum*, intravenous injection of tyrothricin or gramicidin in doses approximating the maximum tolerated dose suppresses the parasitemia (119). Large doses of streptomycin do not suppress the development of blood-induced infections of *P. cathemerium*, *P. lophurae*, or *P. gallinaceum*, although this substance has some inhibitory action on the course of sporozoite-induced infections of the last named parasite (120).

*Metal derivatives.*—Heavy metal derivatives such as mapharsan carbarsone and thiobismol are not effective in the therapy of human malarias (121). The latter drug is without effect upon the course of falciparum malaria, but alters the time sequence of paroxysms in vivax malaria (122) by elimination of half-grown parasites (123).

In the foregoing, nothing has been said of the mechanism of action of antimalarial drugs other than to note that the antimalarial activity of sulfonamides is readily antagonized by *p*-aminobenzoic acid (112, 113, 114). This antagonism indicates that the action of drugs of this class upon plasmodia involves a mechanism similar to that used to explain the antibacterial action of the sulfonamides. Presumably other antimalarial drugs, such as quinine and quinacrine, likewise act by the competitive or noncompetitive inhibition of enzyme systems essential for the life processes of plasmodia. This is indicated by the interesting observation that in parasitized birds the antimalarial activity of these drugs is markedly inhibited by the simultaneous administration of relatively enormous doses of pyridoxine (124). No clue to the mechanism of this unique antagonism has been observed.

Experiments upon the *in vitro* survival of duck erythrocytes parasitized with *P. lophurae* have indicated the possibility that pantothenic acid may be an important factor in the nutrition of malarial parasites (125). Accordingly, the antimalarial activity of several derivatives of *d*-pantoic acid, known to inhibit the use of this metabolite by bacteria, has been examined. All substances of this class so far examined have failed to display antimalarial activity when assayed in ducks parasitized with *P. lophurae*, but a

number of these substances exhibit definite antimalarial activity when assayed in chickens infected with either *P. lophurae* or *P. gallinaceum*. In chickens, the antimalarial action of pantothenic acid inhibitors is largely negated by the simultaneous administration of calcium pantothenate (1, 126).

Several pantothenic acid inhibitors have been found to be equal, or superior to quinine in the suppression of blood-induced infections of *P. gallinaceum* in chicks. In this infection the potency of phenylpantothenone (127) has been found to be equal to that of quinine (1), while the potency of pantoyltauramido-4-chlorobenzene is at least four times that of quinine (126). Interestingly enough, such pantothenic acid inhibitors do not suppress the development of sporozoite-induced infections of *P. gallinaceum* (127).

In the near future, as in the past, it is likely that the search for new antimalarial drugs will proceed on an empirical basis, because so little is known of the metabolic peculiarities of the parasites involved. Some attention has been devoted to an examination of the nature of the enzyme systems operative in malarial parasites, and the action of certain antimalarial drugs upon these enzymes. Because the results of investigations of this nature have recently been summarized elsewhere (128, 129) and because as yet these results have failed to offer any guide to the structural design of new antimalarial drugs, they have not been considered in this review.

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# BACTERIAL METABOLISM

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In 1943, van Niel (1) noted a general shift of emphasis in this field from general and intermediary metabolism towards studies centered around bacterial nutrition. This trend has been very marked during the present year, and, although "growth factors" were the subject of a separate article by Snell (2) last year, there is again much to report. On the other hand there have been comparatively few studies dealing with the end products and intermediate stages of catabolic processes. Detailed consideration of this aspect will therefore be omitted this year since it was covered fully by Barker & Doudoroff (3) in 1946. Furthermore, the more important advances in recent years have been made by the use of isotopic "tracer" elements, and an article by Kamen on this method will be found elsewhere in this volume.[1] Two general reviews (4, 5) include work on the fixation of carbon dioxide by bacteria, and Lipmann (6) has summarized the role of acylphosphates in bacterial metabolism.

Inhibition of growth by substances chemically related to essential metabolites remains an active field of research. The subject has been recently reviewed comprehensively by Roblin (7), and in more general terms by Woolley (8), and will not be dealt with here except when such inhibitors have proved useful tools in particular researches. General aspects of bacterial growth are considered in monographs by Monod (9) and, from the physicochemical standpoint, by Hinshelwood (10). The biological background of our subject is emphasized in Dubos' (11) excellent book. The biochemical activities of marine microorganisms have been surveyed by Zobell (12).

There can, of course, be no real distinction between metabolism and nutrition (though the terms are often used for convenience in a general sense), and this is exemplified by recent progress (reviewed below) towards an understanding of the precise function of several growth factors. An outstanding problem in all branches of biochemistry is that of the anabolic processes of the cell, but an

[1] *Ann. Rev. Biochem.*, **16**, 631–54 (1947).

encouraging beginning has been made with microorganisms, mainly with regard to the nature of intermediate compounds in the synthesis of essential metabolites.

Analysis of the ultimate details of enzyme reactions usually involves the use of isolated enzyme systems. Utter *et al.* (13) have published some helpful general observations concerning their glass-grinding technique of preparing cellfree bacterial extracts. Stumpf & Green (14) give similar information respecting their ultrasonic disintegration procedure. This method, and that suggested (15) by Curran & Evans (shaking with minute glass beads) have not yet been much used in enzyme work but should prove valuable. An observation which may have wider application has been made by Utter *et al.* (16) who find that salts increase the range of substrates oxidized by cells lysed with lysozyme.

Bacteria occur naturally in mixed populations rather than as pure cultures, and more detailed knowledge of their biochemical activities under these conditions is much needed. Recent examples of this type of work include the study, by a new technique, of nitrification in soil (17), and of carbohydrate metabolism in the sheep rumen (18).

As in previous years it has been necessary to restrict the detailed portion of this review to certain topics. The material has been chosen to illustrate general methods of investigation in those fields and it has, of course, been impossible to include every publication.

## Nutrition

Work concerning individual growth factors was reviewed last year (2) and the present review will be concentrated on more general aspects. Lwoff (19) has amplified his views on the relationship of growth factor requirements to evolution amongst microorganisms. Other reviews deal with the utilization of hydrocarbons (20) and the nutrition of lactic acid bacteria (21). Dubos' (22) important observation that rapid, diffuse and abundant growth of tubercle bacilli can be obtained by the addition of surface active substances to the medium should facilitate studies with these organisms. The problem of the obligate anaerobe has again been tackled (23 to 25); synthetic reactions appear to be inhibited by oxygen whilst energy yielding reactions are not.

The importance of the inorganic constituents of a medium has been emphasized by several studies. The correct balance of inor-

ganic salts greatly improves growth and spore yield of the anthrax bacillus on a synthetic medium (26). When the salt concentration of the normal medium is reduced, the ability of *L. fermenti* to synthesize histidine is much increased (27). Photosynthetic purple bacteria require some unusual trace elements (28) which have not yet been fully defined; there are great technical difficulties in this work.

The requirements of several new groups of organisms have been examined. Photosynthetic purple bacteria are able to live autotrophically except for the need of complex microfactors. Hutner (28) has identified these factors as members of the vitamin B group; there is considerable species specificity as to the factors required and several unclassified strains were assigned to species on this basis. Plant pathogens of the genera *Xanthomonas* and *Agrobacterium* will often grow on simple media but some strains require amino acids and/or B vitamins (29).

High yields of fully virulent anthrax spores have been obtained in a chemically defined medium (26); the criterion used throughout was spore yield, so it is not possible to say if there are special requirements for spore formation as opposed to vegetative growth. It is thought that the amino acid mixture finally used might be improved since there is a relation between optimum amino acid nutrition and the concentration of other components of the medium. Thiamine is an absolute requirement, while purines and glutamine are stimulatory. The amino acid and growth factor nutrition of *Cl. botulinum* has also been worked out in detail (30); here biotin is the only essential factor though other B-vitamins and nucleic acid derivatives improve growth. The essential amino acid requirements of *Strept. fecalis* (31) and *L. fementi* (27) have also been reported. Cystine is the best form of sulphur for *Proteus morganii*, though several other compounds, including sodium sulphides, permit fair growth (32).

Acetate has been commonly used as a buffer in synthetic media for *Lactobacilli* and other organisms. Guirard *et al.* (33) have now shown that it has a specific function in stimulating initiation and rate of growth. A concentrate from brewers' yeast has much greater activity than acetate but the nature of the active principle is still obscure. From replacement studies with fatty acids and fat soluble substances, it is suggested that acetate may act as precursor of cell lipoids.

*The folic acid group of factors.*—Many outstanding problems in this field (2) have been greatly illuminated by the publication of the structure of several members of this complex. Analysis of the degradation products of "fermentation" *L. casei* factor led (34) to the synthesis of "pteroylglutamic acid"—N-[4-{[(2-amino-4-hydroxy-6-pteridyl) methyl] amino} benzoyl] glutamic acid—, which proved to have the biological activity of "liver *L. casei* factor" (i.e., active for both *L. casei* and *Strept. fecalis*). The pteroyl radical is defined as *p*-aminobenzoic acid linked via the amino group to the pterin residue. Vitamin $B_c$ from liver and yeast is also identical with this substance (35). The existence of a factor (SLR), active for *Strept. fecalis* (*lactis*) *R* and inactive for *L. casei*, has been known for some time (36 to 38). It has now been shown (34) that pteroic acid (i.e., the molecule without glutamic acid) is active in the same sense, though it is not possible to decide whether the two substances are identical since relative activities are not stated. The fact that conversion of SLR factor to *L. casei* factor (LC) by washed cells of *Strept. fecalis* is greatly increased in the presence of fermentable carbohydrate (39) suggests that the change is an anabolic one. *Strept. fecalis* is presumably able to complete the synthesis of LC factor when given the less complex compound.

Other members of the folic acid group appear to be peptides of pteroylglutamic acid with glutamic acid. Thus vitamin $B_c$ conjugate has been identified as pteroylheptaglutamic acid (35) and there is evidence that "fermentation" LC factor is a pteroyltriglutamic acid (34). The "fermentation" factor has considerable activity for *L. casei* but much less for *Strept. fecalis* (40, 41). It seems possible that the activity of this factor depends on the ability of the organism to hydrolyze it to free pteroylglutamic acid, and that *Strept. fecalis* is less active in this respect. A similar difference in the power of these two organisms to split a naturally occurring conjugate is indicated in another paper (42). An LC factor from yeast was reported earlier (43) as being less active for *Strept. fecalis* than for *L. casei.* If the factor contained some conjugate this observation might be explained along similar lines. Animal tissues are known to contain enzymes which hydrolyze conjugated folic acid; such enzymes are carboxypeptidases (35) and their kinetics have been studied (44).

It is doubtful, however, whether the compounds of known con-

stitution account for all the known activities of members of this group. Liver contains two chloroform soluble factors which can replace folic acid in the growth of *L. casei* and *Strept. fecalis* (45), and whose properties differentiate them from folic acid. Preliminary reports (46, 47) have been given of factors necessary for the growth of *L. casei* which are not identical with natural or synthetic folic acid; analysis of the effect of the natural factor on growth and acid production suggest that this material may be complex (47). Incubation with chicken liver increases the activity of LC factor as measured by *Strept. fecalis* assay (48); this may indicate that the factor preparations used contained conjugate, but other explanations are possible. The increase in this effect caused by pyracin has not been explained. Finally there is evidence (39) that LC factor, as synthesized from SLR factor by bacteria, is less stable than that from other sources.

The relationship of folic acid to sulfonamide action will be discussed later.

## Metabolic Function and Metabolism of Growth Factors

The study of an essential growth factor is not completed by its chemical identification; it is then necessary to discover with what cell processes the factor is concerned and the precise mechanism of its action. Considerable attention has been given to this aspect of bacterial nutrition during recent years, and an attempt will be made to summarize the findings, and to indicate the types of methods which have been used in attacking this problem. Certain factors (e.g., thiamine, nicotinic acid, riboflavin) have been known for some time to be components of important enzyme systems, and this probably represents their principal metabolic function; the facts have been sufficiently covered by earlier reviews in this Journal [e.g. (1)]. Recent progress in this field has been made, in the main, by the following five methods, or by a combination of two or more of them:—

(*a*) Discovery that a component of an isolated enzyme system is identical with a known growth factor.

(*b*) Study of the metabolism of organisms grown on media deficient in the factor in question; followed by detailed investigation of any metabolic process which appears to

have been affected. This method, first used by Peters (49) in studies of vitamin $B_1$-deficient pigeon brain, has proved most valuable when applied to bacteria.

(*c*) Use of substances, often analogues of the factor (antimetabolites), which may specifically inhibit the utilization of the factor.

(*d*) Study of the metabolism of the factor itself and its linkage with other metabolic processes of the cell.

(*e*) The discovery, in growth experiments, that the factor may be replaced by a substance (X) of a different chemical type may suggest that the factor is involved in the synthesis of X or vice versa.

Information concerning the general type of reaction in which a factor is involved may sometimes be obtained indirectly. Thus it has been shown (50) with *Lactobacillus arabinosus* that growth in suboptimal concentration of biotin or pantothenate leads to the production of fewer organisms per unit of lactic acid produced than is the case with suboptimal nicotinic acid. It is therefore suggested that lack of biotin or pantothenate interferes to a greater extent with synthesis of protein than does lack of nicotinic acid, and that the latter is more concerned with glucose breakdown.

*Vitamin $B_6$ group*.—The function of pyridoxal phosphate as a component (coenzyme) of certain amino acid decarboxylase enzymes is clearly established and the evidence [obtained mainly by methods (*a*) and (*b*)] has been reviewed recently by Gale (51). Synthetic pyridoxal phosphate has been purified and evidence given that the phosphoryl radicle is attached to the phenolic group of pyridoxal (52). Further evidence that pyridoxal phosphate is the functionally active member of the $B_6$ group is provided by the finding (53) that pyridoxine, pyridoxal and pyridoxamine are converted to codecarboxylase by growing cultures of all organisms which can use them as a source of $B_6$; cell suspensions can also bring about this change in the case of pyridoxal and pyridoxamine, though with the latter the presence of a keto acid is also needed. In earlier work it was found (54) that nicotinic acid (as well as pyridoxine was necessary for tyrosine decarboxylase formation by *Strept. faecalis*. It has now been shown (55) that nicotinic acid has much less effect on the formation of the apoenzyme, and it is therefore suggested that it is involved, through the energy mechanisms of the cell, in the conversion of pyridoxal to the coenzyme

The intervention of pyridoxal phosphate in amino acid metabolism is not, however, limited to the decarboxylases. Snell (56) suggested that a pyridoxal$\rightleftarrows$pyridoxamine transformation might play a catalytic role in transaminations, and (57) showed that reversible transamination between pyridoxal and glutamic acid occurred on autoclaving. Gunsalus and his colleagues (58) then found that synthetic pyridoxal phosphate functions as coenzyme with cell-free preparations of *Strept. fecalis* R catalyzing transamination between glutamic acid and oxaloacetic or pyruvic acid. The activating effect was observed both with apoenzyme from cells grown in pyridoxine-deficient medium, and with apoenzyme from resolution of the complete enzyme. It was next shown (59) that pyridoxamine phosphate is equally active in this system (though inactive as codecarboxylase), thus strengthening the view that the mechanism of cotransaminase action depends upon the intermediate transfer of amino groups between pyridoxal and pyridoxamine phosphates. Pyridoxal phosphate also appears to be active as coenzyme in some animal transaminase systems though not in all (60 to 64).

Working with cell-free preparations of *Neurospora*, Umbreit *et al.* (65) have found that synthetic pyridoxal phosphate also activates the enzyme system which brings about the direct condensation of indole and serine to form tryptophane (66). A wider role of this factor in amino acid synthesis is possibly indicated by the finding that the growth requirement of *L. arabinosus* for phenylalanine, tyrosine and arginine, and of *L. casei* for aspartic acid, can be replaced by pyridoxine plus carbon dioxide, but not by either alone (67).

It is clear that members of the vitamin $B_6$ group have a general function in amino acid metabolism involving decomposition, interconversion and synthesis.

*Pantothenic acid.*—Using method (*b*) with *Proteus morganii*, evidence has been obtained (68, 69) that pantothenate is involved in the aerobic and anaerobic metabolism of pyruvic acid and possibly other substrates (in particular $C_4$ and $C_5$ dicarboxylic acids, but not glucose); the oxidation of pyruvate to acetate has been especially implicated. McIlwain (70, 71) has attacked the problem by a combination of methods (*b*), (*c*), and (*d*), and by a quantitative examination of the effect of the factor and of a specific "antimetabolite" inhibitor (pantoyltaurine) on the rate and extent of

growth. Using hemolytic streptococci as main test organism he finds that pantothenic acid is itself metabolized provided that glycolysis is taking place simultaneously. Since pantothenate disappearance, but not glycolysis, is inhibited by pantoyltaurine; and since glycolysis by pantothenate-deficient cells is stimulated only slightly by pantothenate; it is concluded that this factor is not involved directly in the glycolytic mechanism. It appears, however, that the metabolism of pantothenate is intimately connected with its growth factor function, because (*a*) the rate of metabolism is similar with growing cultures and cell suspensions, and (*b*) there is correlation between the inhibitory effect of a number of pantothenate analogues on growth and on pantothenate disappearance. McIlwain suggests that the data as a whole are compatible with the view that the coupled metabolism of pantothenate and glucose yields an unknown substance (s) which is essential for growth, and that this reaction is inhibited by pantoyltaurine.

Two findings come under the heading of method (*e*). It has been shown (72) that the requirement of two strains of *Staph. aureus* for tryptophane can be met by pantothenate if glucose is also present (see p. 619). With *Cl. botulinum* growth occurs in a pantothenate-free medium provided that thiamine and choline are both present (73); whether this means that the latter are required for the synthesis of pantothenate or vice versa remains to be determined. It is also reported (74) that threonine synthesis by yeast is aided by pantothenate.

The metabolic role of this factor is not yet clear. It appears to be involved in several systems of rather different types, but more rigid analysis may show that its function is concerned with a key reaction common to these systems.

*Glutamine.*—McIlwain (75) has studied this factor by method (*d*). Glutamine is decomposed by growing cultures of hemolytic streptococci (which require it for growth), and also by cell suspensions when glucose fermentation is proceeding concurrently; the glutamine is hydrolyzed almost quantitatively to ammonia and glutamic acid. The rate of glucose metabolism is almost doubled when glutamine is present, but the products of fermentation are not changed. Although Gale's (76) observation that absorption of glutamine and glutamic acid by gram-positive cocci can only proceed concurrently with an energy yielding process might ac-

count for part of these findings, the stimulation of glycolysis by glutamine remains to be explained. McIlwain suggests that this metabolite plays a part in streptococcal glycolysis, by a mechanism possibly involving an ammonia transfer, and there is some evidence that the hydrolysis of glutamine is correlated with its growth promoting activity. The finding (77) that the sulfoxide of methionine may inhibit the synthesis of glutamine from glutamic acid may provide a useful specific inhibitor for future studies.

*Biotin.*—Winzler *et al.* (78) have shown an interrelationship between biotin and ammonia with *Saccharomyces cerevisiae* which may provide a clue as to the function of this factor. Cells grown in media containing suboptimal biotin showed greatly depressed rates of respiration and fermentation; these rates were increased by the addition of biotin plus ammonia but not by either alone. The assimilation of biotin by deficient cells was found to depend on the presence of glucose and phosphate, and that of ammonia in turn upon the presence of biotin. The suggestion has also been made (79) that biotin may function as a coenzyme of carbon dioxide transfer, since the molecule contains a ureylene structure which can be opened or closed by yeast with loss or gain of carbon dioxide.

*Miscellaneous factors.*—Evidence has been presented that (*a*) *m*-inositol is involved in fermentation by *Cl. saccharobutyricum* (80), (*b*) iron protoporphyrin in the reduction of nitrate to nitrite by *Hemophilus influenzae* (81), and (*c*) strepogenin in the metabolism of glutamic acid by *Strept. fecalis* and *L. casei* (82).

*p-Aminobenzoic acid; mode of action of sulfonamides.*—Since it has been argued by Henry (83) and Sevag (84) that *p*-aminobenzoic acid (*p*-AB) is not a growth factor within the usual meaning of the term, it is necessary to recapitulate the evidence. Twelve bacterial species, representing six genera, were listed in 1945 (85) as requiring, or being stimulated by, this substance; most of them did not grow appreciably without it, and the "blank," if any, was no higher than in studies with other growth factors on similar basal media. It has since been shown that *p*-AB is required by *Leuconostoc mesenteroides* Pd-60 (86) and *Rhodopseudomonas palustris* (28), and stimulates the growth of *Cl. botulinum* (30). Striking evidence that this substance is a true essential metabolite has come from studies of induced mutants. Treatment of several molds (87 to 89)

and *E. coli* (90, 91) with x-rays, U. V. light or mustard gas yields mutants specifically requiring *p*-AB for growth; mutants requiring well-established growth factors are obtained (92) under precisely the same conditions. It is also suggested (83, 84) that *p*-AB cannot be a growth factor since it often reduces growth at higher concentrations. A similar effect (at concentrations above the optimum) is shown by nicotinic acid with *Shigella* (93), thiamine and riboflavin with *Rhizobium* (94) and $\beta$-alanine with *Saccharomyces* (95). The reviewer is unable to see any justification for not considering *p*-AB as a normal growth factor. Many organisms which do not require preformed *p*-AB for growth have been shown to synthesize for themselves material with the same biological activity, and there are analogous findings for all other growth factors (85).

Since the growth inhibitory action of the majority of sulfonamides is annulled in a competitive manner by *p*-AB, it is likely that the elucidation of the metabolic function of this factor will illuminate the basic mode of action of these drugs. Furthermore the sulfonamides may be used as specific inhibitors in such studies. It was suggested (96), as a working hypothesis, that sulfonamides competitively inhibit an enzyme reaction involved in the utilization of *p*-AB. Considerable information as to the nature of this utilization is now to hand, and the interrelationships of *p*-AB with (*a*) the purine bases, (*b*) methionine and (*c*) folic acid are of particular interest.

The *p*-AB requirements of *Acetobacter suboxydans* (97) and some species of *Lactobacilli* (98, 99) are quantitatively reduced, and in the case of *Cl. acetobutylicum* (100) probably abolished, in the presence of purine bases. The concentration required is about a thousand fold that of *p*-AB, and rather better results are usually obtained with a mixture of bases, although adenine alone is almost as active. With *L. arabinosus* and *L. pentosus* adenine, guanine, xanthine and hypoxanthine reduce the amount of *p*-AB required to antagonize sulfonamide (99), while with *Eremothecium Ashbyii* and yeast, adenine itself has about one-tenth the anti-sulfathiazole activity of *p*-AB (101). There is also evidence (102) that yeast grown in the presence of sulfathiazole has a diminished nucleic acid content. Guanine and xanthine are sulfonamide antagonists (*E. coli*) but only in the presence of methionine (103). A number of other studies have implicated this amino acid. At high concentra-

tions it can replace the *p*-AB requirement of certain *Lactobacilli*, and has antisulfonamide activity with *L. casei* (99) and *E. coli* (103 to 105), though only over a limited range of drug concentration. Other amino acids have slight activity (103, 106) but only if methionine is present (103). Harris & Kohn (104) suggest that low concentrations of these drugs inhibit the synthesis of methionine, and that this synthesis, as well as others important to the cell, depends on the utilization of *p*-AB.

Shive & Roberts (107) find with *E. coli* that the critical ratio sulfonamide/*p*-AB is raised threefold if methionine is present and a further threefold if guanine or xanthine is also added. Similar experiments with *L. arabinosus* gave a tenfold increase in the presence of adenine, and it is deduced that purines and methionine are products of reactions involving *p*-AB. These interrelationships have been much clarified by recent work with an induced mutant of *E. coli* requiring *p*-AB. It is found (90) that this factor may be replaced by amino acid mixture (of which methionine is the most important constituent) plus adenine, guanine, xanthine and relatively high concentrations of thymine. Growth on this meduim is subcultivatable and highly resistant to sulfonamides; and it is concluded that, with *E. coli* at least, *p*-AB functions in the synthesis of the purines, thymine, and one (probably methionine) or more amino acids. It will be recalled that there is also evidence (2) that folic acid may be concerned with the synthesis of purines and thymine.

The pteroylglutamic acid molecule (see p. 608) contains a *p*-AB moiety, and this suggests at once that *p*-AB is required for the synthesis of this factor. The production of "folic acid" by mixed cultures from the fowl intestine was found earlier (108) to be stimulated by *p*-AB, while growth of *Mycobact. tuberculosis* on a medium containing *p*-AB led (109) to the production of material with the properties of vitamins $B_{10}$ and $B_{11}$ (members of the folic acid group).

If the only function of *p*-AB is to act as a precursor for folic acid synthesis it might be expected (*a*) that organisms requiring *p*-AB would grow if given an equivalent concentration of folic acid (providing the latter can be absorbed by such organisms and information on this point is lacking), and (*b*) that folic acid would have antisulfonamide activity of a noncompetitive type (since the product of the inhibited reaction is added). Synthetic pteroylglutamic

acid, however, is inactive as growth factor for the mutant *E. coli* requiring *p*-AB (90) and is less active than *p*-AB (never more than 4 per cent) for several other organisms (110); it is not however certain (see p. 608) that the synthetic substance is the only one of this chemical group required by bacteria. Inhibition by sulfonamides of the growth of *Strept. fecalis* (Ralston),[2] *L. arabinosus* and several enterococci is antagonized noncompetitively by pteroylglutamic acid at a concentration as low as that which promotes growth of organisms requiring this factor, and it is concluded that the synthesis of pteroylglutamic acid from *p*-AB is the point of sulfonamide inhibition with these organisms (111). This phenomenon is not, however, obtained with *E. coli*, *Staph. aureus* or *Diplococcus pneumoniae*. Noncompetitive antagonism is also found (110) with *Streptobacterium plantarum* 10 S at a pteroylglutamic acid level of the same order as that promoting growth (50 to 100 times that of *p*-AB). Interference by these drugs in the synthesis of folic acid is also suggested by observations (99, 111) that organisms requiring preformed SLR or LC factors are particularly resistant to sulfonamides, and (112) that the production of folic acid by growing cultures of *E. coli* is inhibited by sulfanilamide.

*p*-Aminobenzoylglutamic acid is reported to be 5 to 10 times more active than *p*-AB as sulfonamide-antagonist with *Streptobact. plantarum* 10 S (113). This is not the case with other organisms (114, 115) but the observation has now been repeated (110) with the 10 S strain and at several levels of sulfanilamide and sulfathiazole concentration; the relationship is, however, a competitive one, so it is presumably not the synthesis of this moiety of folic acid which the drugs inhibit.

There is thus strong evidence that *p*-AB is concerned in the synthesis of folic acid, purines, thymine and methionine, and that sulfonamides interfere with these functions. In the case of the nucleic acid derivatives, folic acid is possibly the primary intermediate, but there is as yet no hint as to the mechanism of the effect of *p*-AB on methionine anabolism. This review is of necessity restricted to the relationship between the metabolic function of *p*-AB and the action of sulfonamides, but if these drugs do interfere (as seems probable) with the synthesis of such key substances

[2] This organism is not *Strept. fecalis* R.

to the cell as the purines and pyrimidines, it is reasonable that many other cell processes should be secondarily affected. Thus the effect of these drugs on respiration (83, 84) may arise from the fact that adenine is involved in the structure of the respiratory coenzymes. Two groups of workers (115, 116) have suggested that sulfonamides may inhibit synthetic reactions leading to proteins and peptides; such processes may well involve both nucleic acid and methionine.

## Problems of Biosynthesis

It is generally accepted that the substances recognized as growth factors for particular microorganisms are essential for the growth of most microorganisms, but that some organisms can synthesize a given factor whilst others cannot (19, 117). In a number of organisms requiring the same factor, the power to synthesize may have failed at different stages of the series of reactions by which the synthesis is accomplished, and the provision of intermediates above the point of failure will permit the synthesis to be concluded and the organism to grow. Thus organisms are known which require substances of increasing complexity (ammonia, nicotinic acid, nicotinamide, nicotinamideriboside) for the synthesis of the phosphopyridinenucleotide coenzymes [for references see (137)]. Similar evidence as to the path of synthesis can often be obtained with a single organism if a number of mutants, each requiring ultimately the same essential metabolite, are available for study (92). Here again the mutants may fail at different stages of the synthetic process. This approach is made more practicable if the slow natural mutation rate can be increased, and the outstanding work of Beadle and co-workers (92) with x-ray or U. V. induced mutants of *Neurospora* has proved the value of the technique. A recent example (118) concerns the biosynthesis of thiamine. It seems only a matter of time before equally fruitful results are obtained with bacteria. Biochemical mutants needing a variety of essential metabolites (including amino acids) have been obtained by the action of x-rays and U. V. light (91, 119 to 121) or bacteriophage (122) on *E. coli*, and such mutants appear to be stable (121). The induction of mutants in *Neurospora* by mustard gas (123) may also prove applicable to bacteria. A rapid method of

separating such mutants from normal organisms has been described (91).

When an exacting organism is grown in the presence of the required growth factor it sometimes happens that the intermediate compound corresponding to the point of failure of the synthetic mechanism accumulates in the system. A nicotinamide-requiring mutant of *Neurospora* (124), growing in the presence of this factor, produces a substance which promotes the full growth of a similar, but genetically distinct, mutant. The substance is presumably a precursor in the synthesis of nicotinamide and there is evidence that it is an oxypyridinecarboxylic acid. Accumulation of precursor may also occur (125) when growth is limited by specific inhibitors of the utilization of a metabolite. Such growth inhibitions (e.g., by metabolite analogues) may, if competitive in nature, yield further information; the addition of the product of the inhibited reaction will promote growth irrespective of inhibitor concentration (126), or, if the inhibitor affects also other reactions, a higher concentration may be required to suppress growth (127).

*Synthesis and breakdown of tryptophane.*—The case of tryptophane illustrates well the general methods outlined above. The finding that the trytophane requirement of *Bact. typhosum* could be met by indole (128), and of certain species of *Lactobacilli* by indole or anthranilic acid (129), suggested that these two substances are intermediates in the synthesis. Studies with mutants of *Neurospora* (66) confirmed this idea and led also to the discovery that, with this organism, the final stage in the synthesis is a direct condensation of indole and serine; pyridoxal phosphate has now been shown (65) to be involved in the condensation mechanism. Tatum & Bonner (66) also found some evidence of tryptophane formation when *E. coli* was incubated with indole and serine. Fildes (125) was unable to confirm this (possibly owing to strain difference—the organism also breaks down tryptophane to indole, so the balance may be delicate), but obtained evidence by other methods for the role of indole and serine as intermediates with bacteria. Using a strain of *Bact. typhosum* trained to grow without either indole or tryptophane, and indoleacrylic acid as a specific inhibitor of tryptophane synthesis from indole (126), he found an accumulation of indole when growth was partially suppressed by the inhibitor; furthermore growth inhibition was much reduced if

serine were added. Schweigert *et al.* (130), using an amino acid medium not containing serine, found that indole has only a quarter of the growth-promoting activity of tryptophane for *L. arabinosus*, but that this is doubled if serine is also added. Since the increased activity still falls short of that of tryptophane, it is thought that another substance (s) (shown to be present in hydrolyzed casein) is also involved. The action of pantothenate (plus glucose) in replacing the trytophane requirement of two strains of *Staph. aureus* (72) may reflect a function of this factor at an earlier step in the synthesis.

The mechanism of the degradation of trytophane to indole has been an outstanding problem for many years. In recent work (131), the suggestion that β-*o*-aminophenylacetaldehyde is an intermediate (132) has been criticized on the grounds that dimedon inhibits spontaneous indole formation from this substance, but not from tryptophane by bacteria. Tatum & Bonner (66) thought that breakdown might occur by a direct split to indole and serine (i.e., the synthetic reaction in reverse) and found indole production by *E. coli* to be slowed down by serine. Baker *et al.* (131) have extended their earlier work (133) on essential structural conditions for the tryptophane-indole reaction and find support for their suggestion that the alanine side chain is removed as a whole. Strong direct evidence for this mechanism has come recently from the same laboratory (134, 135): in the presence of mepacrine, washed cells of *E. coli* produce initially some indole without concurrent oxygen uptake, and during this period alanine (but not serine) can be detected by partition chromatography.

*Other essential metabolites.*—A number of papers presenting evidence for possible intermediates in the synthesis of several growth factors were reviewed last year by Snell (2); such evidence was based mainly on the capacity of substances to promote growth in the absence of the factor in question. Specific "antimetabolite" growth inhibitors are being used increasingly in this connection. Inhibition of growth of *E. coli* by β-hydroxyphenylalanine and thienylalanine (136) is counteracted by phenylalanine and also, though less effectively by tryptophane, and it is suggested that the latter is involved in the synthesis of phenylalanine; in the case of thienylalanine, however, other workers (137) find tryptophane as effective as phenylalanine. Growth inhibition (*E. coli*) by *p*-hy-

droxyaspartic acid is completely annulled by aspartic acid, while pantothenic acid and β-alanine raise the concentration of inhibitor required to suppress growth (127). It is concluded therefore that this inhibitor prevents at least two essential functions of aspartic acid, one of which is to act as precursor (by decarboxylation) of the β-alanine moiety of pantothenic acid; the nature of the other function (inhibited at higher concentrations) is not known. The finding (138) that pantothenic acid antagonizes the growth inhibitory effect of salicylate, and that pantoate is more active in this respect than pantolactone, indicates that pantoate rather than the lactone is the precursor of pantothenate: this view is supported by other work on the relative activities of these substances in replacing pantothenate as a growth factor (139, 140).

Certain growth factors are known to be components of enzyme systems and recent work with "antimetabolites" may throw light on the mechanism of formation of the ultimate complete enzyme. The utilization of diphosphothiamine for growth of *L. fermentum* and *Pencillium digitatum* is more susceptible to inhibition by pyrithiamine than is that of free thiamine, and these results (141) are interpreted as indicating that thiamine is attached to the apoenzyme before phosphorylation. The effect of the same inhibitor on the growth of thiamineless mutants of *Neurospora* is overcome more effectively by pyrimidine plus thiazole than by thiamine itself (118). It is considered possible therefore that linkage of the moieties may occur only after union with the enzyme protein. In the case of riboflavin, analogous findings with *L. casei* using lumiflavin as inhibitor suggest its combination with apoenzyme before conversion to riboflavin phosphate or flavin-adenine-dinucleotide (142).

The fact that both boron and *m*-inositol (though not succinic acid) reverse the inhibitory effect of malonate on fermentation by *Cl. saccharobutyricum* may indicate that boron is a coenzyme in the synthesis of inositol (80).

*Control of synthetic mechanisms.*—McCarty (143) has contributed an excellent review on the transformation of pneumococcal types. The identification of the substance controlling the synthesis of the polysaccharide of Type III *Pneumococcus* as a desoxyribonucleic acid has been made more rigid by work (144) with a highly purified desoxyribonuclease. The value of this enzyme as a specific

reagent is increased by the fact that its activation by magnesium is inhibited by citrate while that by manganese is not. By using citrate to inhibit the desoxyribonuclease liberated from pneumococci themselves on lysis, McCarty & Avery (144) were able to improve the yield of extracted Type III transforming factor fivefold, and have isolated similar factors from Type II and VI organisms. These were again desoxyribonucleic acids. It is pointed out (144) that only a small proportion of the molecules of a preparation are active as transforming principle since rough (R) strains give a similar desoxyribonucleic acid fraction but without activity in this sense. This work must surely be a classic in the technique of elucidation of structure of large molecules by enzymic analysis. Boivin *et al.* (145) have shown the presence in *E. coli* of a similar factor controlling the synthesis of polysaccharide, and able to bring about transformation of Types via the rough form. There is evidence that the factor is a polymerized thymonucleic acid and that transformation involves also loss of ability to ferment sucrose.

*Miscellaneous.*—The phenomenon of oxidative assimilation has been fully covered by Clifton in a recent review (146). Such assimilation involves the synthesis of substances of higher energy content, and Clifton favors the concept that such changes may occur by the intimate coupling of anabolic and catabolic reactions without energy transfer from a materially independent system. The synthesis of polysaccharides from sucrose by cell free enzyme preparations of bacteria was dealt with last year (3); in a recent paper (147) the kinetics of a more potent preparation from *Leuconostoc mesenteroides* are described.

## Factors Associated with the Toxicity and Virulence of Bacteria

*Isolation and chemical nature of bacterial toxins.*—The crystallization of the toxin of *Cl. botulinum* Type A has been achieved by two groups of workers in the same laboratory using different methods. The procedure of Lamanna *et al.* (148, 149) involves shaking with chloroform as an important step: this removes inactive protein and shifts the critical pH of acid precipitation of the toxin; the salt concentration during purification is also an important factor in increasing the stability of the toxin. Abrams *et al.* (150) use sodium sulfate and acid precipitations. In both cases the

crystalline toxin has the properties of a protein of the globulin type. The toxicity remains unchanged on recrystallization and there is further evidence of the homogeneity of the protein from sedimentation, electrophoresis and absorption spectrum studies (148, 150 to 152). The mol. wt. is computed to be $9 \times 10^5 - 1.1 \times 10^6$ (151, 152) and a preliminary analysis by microbiological assay methods indicates nothing unusual in the amino acid composition of the protein (153). The crystalline toxin is the most poisonous substance known, the toxicity for mice being *ca.* $2 \times 10^8$ $LD_{50}$/mg. nitrogen, or $32 \times 10^9$ $LD_{50}$/gm. dry toxin; on the basis of a mol. wt. of $9 \times 10^5$ it is calculated (151) that a single mouse $LD_{50}$ is equivalent to $2.1 \times 10^7$ molecules.

Good growth and toxin production by *Cl. botulinum* Types A and B on media of known composition have also been reported (30). With Type A the titer of toxin was one-tenth to one-twentieth of that obtained on the complex medium used for the isolation of the crystalline toxin.

A second toxin obtained in crystalline form is that of *Cl. tetani.* Pillemer *et al.* (154) using a method (155) in which methanol, under rigidly controlled conditions, is used to precipitate in turn active and inactive materials, finally isolated a crystalline toxin of potency 5 to $7.5 \times 10^7$ mouse M.L.D./mg.nitrogen. The parent high titer toxic culture filtrate was obtained with a poorly sporing variant of *Cl. tetani* (156) on a complex medium (157). The product has constant biological activity on recrystallization and has the general properties of a protein, but no further data concerning its homogeneity have yet been published.

The isolation of these two toxins in crystalline form and a high state of purity will greatly facilitate future investigations concerning their mode of action. The extremely high order of biological activity coupled with their protein nature is suggestive of the possibility that they may be enzymes.

There have also been many reports (too numerous to give in detail here) of progress with other toxins and related factors. Such progress has been mainly in regard to suitable media and conditions for high titer toxin production, and with partial purification of the active principle. Although concentrates have often shown protein properties, it would be premature to draw conclusions as to their chemical nature. There appear to be no special factors for

toxin production (as opposed to growth) when staphylococcal enterotoxin is produced on a chemically defined medium (157a).

*Enzymic nature of certain toxins of* Cl. welchii.—It is now five years since Macfarlane & Knight (158) demonstrated the presence of a lecithinase enzyme in preparations of the α-toxin of Type A *Cl. welchii* and obtained strong evidence for the identity of this enzyme with the actual toxin. In the course of that work it was found that protein-free extract of horse muscle contains a factor which stimulates toxin (and enzyme) production. Rogers & Knight (159) have now purified this factor and find the properties of active fractions suggestive of an amino sugar; glucosamine and *N*-acetylglucosamine proved to have activity equivalent to 30 per cent of that of the factor. The iron concentration of the medium became critical as the factor was purified. A general relationship between *in vitro* α-toxin or lecithinase production and virulence of the organism is brought out by recent distribution studies (160, 161).

Following up an observation (162) that incubation with *Cl. welchii* Type A filtrates causes rapid disintegration of muscle, and that this is due (163) to destruction of the reticulin scaffolding, Oakley *et al.* (164) have confirmed the presence of a collagenase enzyme in such filtrates and worked out an ingenious method of assay. Since the enzyme is immunologically distinct from other recognized components of the toxin complex, and may be responsible for the pulping of muscle in human gas gangrene, the authors have christened the enzyme, "κ-toxin."

*Hyaluronidase* (*spreading factor, invasin*).—The presence of the mucopolysaccharide, hyaluronic acid, in skin, and of the enzyme, hyaluronidase, in pathogenic bacteria, has led to the concept (165, 166) that hyaluronidase plays an important role in bacterial invasion by depolymerizing the mucoid ground substance of connective tissue. If this is the case it seems that invasive power is not a controlling factor in determining overall virulence of an organism, since recent studies with many strains of staphylococci (167, 168) and *Cl. welchii* (160, 161) have failed to show a correlation between virulence and hyaluronidase production. Haas (166) has investigated the defence mechanism of the host against invasin (hyaluronidase) and has shown the presence in normal blood plasma of an enzyme (anti-invasin I) which destroys in-

vasin; further enzymes in bacteria (proinvasin I), and in plasma (anti-invasin II), destroy anti-invasin I and proinvasin I respectively, and it is suggested that the quantitative balance of these enzymes in the host-bacterial system determines whether or not invasion will occur. Several detailed studies of the kinetics and other properties of hyaluronidase have been made (169 to 171). Enzyme production on a simplified medium has been obtained, though some organisms require complex factors; and the enzyme is adaptive with some organisms but not with others. Although enzymes from various sources all hydrolyze the substrate with liberation of reducing sugars and acetylhexosamine, there is considerable variation, according to the source, in the degree of hydrolysis and molecular size of the product, and this is reflected in the differing abilities of such products to promote adaptive formation of the enzyme: it seems possible that hyaluronidase is a complex of enzymes. Activity against other substrates (e.g., chrondroitin sulphuric acid) also varies with the source of the enzyme.

*Bacterial pyrogen.*—Pyrogens from several organisms growing on synthetic media have been concentrated to an activity of 0.01 μg./kg. (rabbit). The chemical properties of the purified materials, which are protein-free, suggest that they may be aminopolysaccharide in nature (172).

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# USE OF ISOTOPES IN BIOCHEMICAL RESEARCH: FUNDAMENTAL ASPECTS

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A discussion of isotopic tracer methods and applications in biochemical research last appeared in these *Reviews* in 1940 (1). Since then no less than 450 separate references have accumulated in these pages under the headings of biological oxidations and reductions, intermediary metabolism of carbohydrates, fats, proteins, amino acids, nucleic acids, nucleoproteins, phosphorus compounds, sulfur compounds, photosynthesis, nonoxidative enzymes, etc. This large literature compares favorably with that in the prewar era despite the considerable constraints imposed both on isotope supply and research time.

It is quite obvious that while tracer methodology already is well established in the biochemical repertoire, its further proliferation into the general field will begin to accelerate markedly as a result of two factors new to the scene since 1940. The first of these is the emergence of the uranium pile reactor as a bulk neutron source of hitherto unimagined intensity making possible general distribution of all neutron-produced radioactive isotopes (2) notably the important long-lived isotopes of hydrogen ($H^3$)[1], carbon ($C^{14}$), phosphorus ($P^{32}$), and sulfur ($S^{35}$). The second factor is the expansion in commercial production of the rare stable isotopes, $H^2$, $C^{13}$, $N^{15}$, and possibly $O^{18}$. It appears that a complete armamentarium for the fullest possible exploitation of tracers is very close to realization for biochemists.

No less important is the successful engineering of assay equipment which now makes possible marketing of commercial machines adequate for most contingencies in the measurement of both radioactive and stable isotopes. A list of manufacturers of instrumentation for assay of radioactive isotopes is obtainable from the Manhattan District, Isotopes Division, Oak Ridge, Tennessee. The price ranges for radioactive assay instrumentation are all well within the budgets of most research institutions. Apparatus for stable isotope assay remains somewhat expensive, but

[1] $H^3$ while produced in large quantities is not available at this time.

those actively engaged in engineering precision mass spectrometers are reassuring with regard to the prospect of providing cheaper machines in the near future. Data and instructions on production and measurement of isotopic tracers have appeared recently (3).

In attempting a review at this time the writer has been influenced by the notion that material already presented in sufficient detail should not be duplicated. This infers only token coverage of tracer applications. In place of such material a discussion of basic methodology, including some aspects not often discussed hitherto, is indicated. In particular, emphasis on the nature and validity of labeling techniques with implications for future research appears necessary. For recent examples of articles which treat adequately a certain general range of tracer applications the reader is referred to the review of carbon isotopes in the study of intermediary metabolism by Buchanan & Hastings (4) and to the well-integrated survey of carbon dioxide fixation in relation to the tricarboxylic acid cycle by Wood (5).

*Basic considerations.*—Tracer methodology is based on the general validity of two facts: (*a*) isotopes of an element are identical in chemical behavior, (*b*) isotopically complex elements always exhibit constant isotopic composition in the natural state. Taking up the constancy of isotopic composition first, it should be remarked that minor isotopic fluctuations occur in the elements hydrogen, carbon, oxygen, and possibly potassium. A detailed consideration of the evidence for and extent of such fluctuations in biological specimens has appeared recently (6). Such fluctuations are important in that they determine the ultimate precision of work with rare stable isotopes. Waters of biological origin are reported to show variations in density up to 3 parts per million (7). Thus, the normal isotope ratios in hydrogen and oxygen may vary by several per cent. Similarly $C^{13}/C^{12}$ ratios fluctuate significantly, the lighter isotope being concentrated preferentially in many animal and plant sources relative to a standard sodium bicarbonate sample (8). The maximum uncertainty appears to be no more than $\pm 0.005$ in the normal $C^{13}/C^{12}$ ratio usually taken as 1:100. This is nearly the limit of precision for most good mass spectrometers. There is no evidence that any marked deviations occur in different specimens from the same organism (9, 10) although careful examination for isotopic effects in hydrogen and oxygen using the more refined methods of density assay still remains to be made.

At any rate, while isotopic fluctuation appears to be outside the range of sensitivity of present methods, it must be borne in mind that they may render futile much further refinement in assay.

The chemical identity of isotopes is maintained in organisms for all elements with the one serious exception of hydrogen. The extreme mass ratios of deuterium ($H^2$) and tritium $H^3$) introduce definite differences both in ultimate equilibrium values as well as in reaction rates for systems involving hydrogen transfer. In most tracer research with the hydrogen isotopes the major concern is with labilization of carbon-hydrogen bonds. The C-$H^1$ bond is weaker than the C-$H^2$ or C-$H^3$ bond by virtue of the higher zero point energy for the lighter isotope. The activation energy is lower for the same reason. Protium ($H^1$) is more easily activated than $H^2$ or $H^3$ and is more rapid to react in transfers from carbon to carbon. The difference in activation energy for the hydrogen isotopes is not negligible, i.e., it may vary from 600 to 1600 calories as in certain photochemical reactions with chlorine (11). Hydrogen transferring enzymes are sensitive to small gradations in activation energy, so that isotopic differentiation can occur in enzyme catalyzed reactions invalidating the use of $H^2$ or $H^3$ as hydrogen tracers.

An example of uncertainty introduced in this fashion has been provided in researches devised to study the role of chlorophyll in photosynthesis (12). The participation of chlorophyll as a hydrogen donor in a reversible redox system was studied by carrying out photosynthesis with green algae immersed in an aqueous medium containing tritium as $H^1H^3O$. Involvement of chlorophyll in a reversible oxidation process would have resulted in the appearance of tritium in chlorophyll, because hydrogen atoms released and taken up in each cycle might be expected to equilibriate with aqueous hydrogen ion. Although the conditions of the experiment were such that each chlorophyll molecule in the algae must have participated in at least 100 cycles, no tritium ($<1$ per cent theoretical) was found in the chlorophyll after extraction and purification. Loss of tritium from chlorophyll by exchange reactions during purification was excluded in control experiments with pure chlorophyll suspended in $H^1H^3O$.

However, it could not be concluded that chlorophyll underwent no cyclic participation in hydrogen transfer during photosynthesis because, as the authors pointed out, there existed the

possibility of isotopic differentiation whereby no labeled hydrogen followed protium into chlorophyll. Thus, there might be imagined a scheme in which photoactivated chlorophyll ($G^*H_2$) could donate hydrogen to a substance A, i.e.,

$$\text{Light} + GH_2 + A \rightarrow G^*H_2 + A \rightarrow GH + HA.$$

The chlorophyll radical could be reduced back to $GH_2$ in a thermal reaction which might have an activation energy with protium two kcal. less than with tritium. Such a difference in activation energy would lead to a specific reaction rate for the protium reaction some twenty times greater than for the tritium reaction.

In other researches such doubts appear less plausible. Thus, when the oxidation of fumaric acid with alkaline permanganate in tritium-labeled water was studied, it was found that the stably-bound hydrogen of the resultant formic acid contained no tritium (13). The formic acid was known to arise from the methine carbons (13). It was concluded that during this oxidation the hydrogen attached to the middle carbons of fumaric acid was not labilized because such labilization would result in ultimate appearance of tritium in the C-H portion of formic acid by equilibration with hydrogen ion of the aqueous medium. Isotopic differentiation could be ruled out because there exist numerous experiments which show that isotopic differentiation is not operative under conditions similar to those obtaining in the reaction system discussed. Thus, to cite a recent example, when isobutane is alkylated with 2-butene in the presence of tritium sulfuric acid, the hydrogen of the alkylate is found to contain an amount of tritium corresponding to complete randomization (14). Further discussion of isotopic exchange reactions involving hydrogen is deferred to later sections.

Isotopic differentiation in elements other than hydrogen is negligible because mass ratios are very close to unity.

*Basic limitations in use of isotopic tracers.*—While the above mentioned difficulty arising from isotopic differentiation in the hydrogen isotopes represents a special type of limitation, there are a number of more general limitations which have arisen to plague isotope researches and which may be mentioned briefly.

The quantitative calculation of metabolic turnover as evidenced by variations in isotopic content of labeled metabolites depends on a number of factors which are not easy to assess. It is required that perfect mixing of labeled ingested material and simi-

lar material of endogenous origin be attained. It is also necessary to establish quantitatively the isotopic contents of whatever precursors are postulated in the process being studied, and to relate such variables to the isotopic content of the metabolite isolated. Calculations are available based on the assumptions that a steady state is set up with formation and disappearance of metabolite being equal, and that no discrimination occurs between newly synthesized metabolite and the same metabolite present from the previous history of the system (15). An instructive application of such considerations to the problem of turnover rate of plasma phospholipids should be consulted (16).

Ambiguities in the intepretation of isotopic dilution experiments designed to reveal mechanisms of formation of end products or intermediary metabolites from a given labeled substrate are not infrequent. A good example is the difficulty which arises when it is endeavored to ascertain how succinate is produced from labeled acetate ($CD_3 \cdot COOH$) in acetate oxidation by yeast (17). Direct condensation of acetate to succinate would lead to the incorporation of four deuterium atoms, stably linked, in succinate. On the other hand, formation of succinate via the tricarboxylic acid cycle is also possible (18) and, if a symmetrical molecule such as citrate is excluded as an intermediate, the resultant succinic would contain two deuterium atoms. Experimentally, the succinate isolated contains two stably-bound deuterium atoms. One may interpret such a result as confirming the postulate that the tricarboxylic acid cycle is operative, or one may equally well invoke dilution of succinate by intervention of reversible equilibria, such as succinate-fumarate, so that the four deuterium atoms originally incorporated via direct condensation from acetate are diluted out, two deuterium atoms resulting in the final product (5). It is evident that deuterio-labeled acetate is not useful in deciding between these alternative mechanisms without considerably more supporting knowledge of details such as relative rates of diluting reactions involved in the tricarboxylic acid cycle, and the magnitude of endogenous reactions contributing unlabeled succinate.

There may be interpolated at this point a few remarks about the stability of carbon-hydrogen linkages since the use of the hydrogen isotopes as auxiliary carbon labels will be discussed at some length later. It is well known that the carbon-hydrogen link is stable toward exchange with aqueous hydrogen ion so long as

the hydrogen atom is not in close proximity to negative groups such as carbonyl, nitrilo, etc. Acidic hydrogen is rapidly replaceable, and reversible equilibria, such as enolization, which promote the formation of acidic hydrogen must be avoided. This subject has been treated at some length in the literature, and a large bibliography on the stability of various carbon-hydrogen linkages as a function of molecular environment is available (19). The important point to be made is that deuterium linked to carbon by a bond stable with respect to exchange *in vitro* with water can be considered stably bound as far as direct exchange *in vivo* is concerned. Removal of deuterium from carbon-deuterium linkages which are stable occurs only by chemical processes (19).

Limitations deriving from somewhat more basic aspects of tracer methodology are those dependent on the physical nature of tracers. The first of these is the dilution range available for tracer studies. The dilution range depends on the concentration of isotope available or permissable, constancy of normal isotopic composition in the element studied, and precision of assay. Deuterium, among the stable isotopes, again constitutes an exceptional case. Deuterium oxide in high concentrations is a respiratory poison (20, 21). Use of deuterium oxide is restricted to concentrations such that the equilibrium value reached in circulating plasma is at a level of 2 to 5 per cent. This practice derives from the observation that dehydrogenases cannot transport deuterium as rapidly as protium in biological oxidations. Thus, to cite one example, glucose fermentation by yeast is nine times as slow in pure $D_2O$ as in ordinary water (22). Such considerations obviously do not apply to deuterium when it is used as an auxiliary carbon label.

The upper limit of dilution for stable isotopes is determined by the magnitude and fluctuations in normal isotopic content and by the precision of assay. Thus, in $C^{13}$ the normal isotopic ratio $C^{13}/C^{12}$ appears to be essentially invariant yielding a value by mass spectrometer assay of $0.0110 \pm 0.0002$ (8). It has been indicated that there exists the possibility of isotopic fluctuation effects just beyond the limit of precision for mass spectrometer assay. Assuming that the precision given is an ultimate limit, it is seen that a sample of 20 per cent $C^{13}$ cannot be diluted more than one-hundred-fold if a precision of $\pm 5$ per cent is to be attained. For deuterium, the normal isotopic concentration is 0.02 per cent and this value can be checked within 5 per cent by mass spectrometer assay. Conse-

quently 20 per cent deuterium can be diluted at least a thousand-fold in precision assay.

The radioactive isotopes are limited in ultimate concentration only by possibility of radiation damage to the organism. It is impossible to make comprehensive statements of general validity on this point.[2] The dosage limits are affected by a number of ill-defined factors such as the effect of selective irradiation consequent on localization in tissues, the quality of the radiations, and the enormous variability in response to radiation dependent on whether a single ionization event or many are required to initiate a chain of abnormal reactions (23). Little systematic data on radiation effects using relatively low radiation dosage is at hand. In general, it appears that processes such as respiration, glycolysis, and nitrogen metabolism are not altered significantly even by large doses of radiation (24). However the effect of localization of radiation attendant on selective concentration of radioactive isotopes cannot be assessed reliably from studies with external sources of radiation. Thus, although general functional activity may not be altered by a given dose, it is possible to affect regulatory functions with the same dose if the radiation happens to be concentrated on a structure such as a chromosome.

With tracer concentrations ordinarily employed there do not appear to be appreciable physiological effects. Some data is available which indicates roughly the radiation levels which should not be exceeded. In studies on the permeability of *Nitella* to sodium ions, using $Na^{24}$, one millicurie equivalent of beta radiation per liter is reported as a limit above which significant radiation effects can be observed (25). However, for somewhat higher radiation levels in studies on yeast metabolism (8 to 10 microcuries $P^{32}$ per cc. of dense cell suspensions) no disturbance in fermentative activity is noted (26). Such specific activities are in excess of the values recommended for use with humans as based on a tolerance limit of 0.1 roentgen body radiation daily (27). However, the human tolerance limit at the clinical level has no relation to the dosage limits available for biochemical work with extracts or microorganisms.

The occurrence of both stable and radioactive tracer isotopes for the same element leads to the suggestion that parallel experi-

[2] An integration of present knowledge has appeared recently in the publication, *Actions of Radiations in Living Cells*, by D. E. Lea (Cambridge University Press, 1946).

ments with the two types of label be devised to reveal radiation effects directly. Thus, in prosecuting researches with $C^{14}$ occasional repetitions using the stable isotope $C^{13}$ can be performed to check abnormalities in metabolic activity induced by radiation. Alternatively, experiments employing graded concentrations of $C^{14}$ can be used to provide information on the radiation level necessary for divergences from results obtained with low tracer concentrations. It is doubtful that concentrations sufficient to cause radiation effects will ever be necessitated in most tracer work.

With these reservations, radioactive tracer isotopes provide much higher dilution ranges than those available with the rare stable isotopes. $C^{14}$ in a concentration of ten atom per cent corresponds to an activity of 0.55 millicuries per milligram based on the half-life of 4700 years recently quoted (28). The lower limit with the usual thin-window Geiger Muller tube which can be measured in a reasonable time (30 minutes) corresponds to approximately $1 \times 10^{-8}$ millicuries so that a dilution range of $5 \times 10^{7}$ is available. Unfortunately the total quantity of such samples is no more than a few milligrams so that in most practical applications considerable dilution will be encountered before the carbon is in a proper chemical combination for biochemical research.

In conclusion there remains to be discussed the limitations in the use of radioactive isotopes occasioned by the phenomenon of nuclear isomerism. A given unstable nucleus may exist in two isomeric forms which exhibit different radiocative properties. These two forms may be genetically related, one decaying to the other which in turn decays to a stable nucleus. As an example, $Br^{80}$ exhibits two half-lifes, 4.4 hours and 18 minutes, due to the existence of isomeric nuclei (29). The 4.4 hour form decays by gamma ray emission to the 18 minute form which decays in turn by beta ray emission to the stable nucleus $Kr^{80}$. In $Co^{60}$, two isomers are found (30), one with a half-life of 10.7 minutes, the other with a half-life of 5.3 years. In this case most of the 10.7 minute isomer appears to decay to the 5.3 year isomer although a few per cent of the 10.7 minute disintegrations proceed directly to the stable nucleus $Ni^{60}$, as do all the disintegrations of the 5.3 year isomer (31).

Matters are further complicated by the existence of radioactive chains in which an element of a given atomic number is transformed by disintegration into an element of atomic number one unit higher which is also radioactive. This process may continue

several times, as in the fission product nuclei. An example of a two-membered chain is $Ca^{49}$ which not only exists in two isomeric forms but decays with a half-life of 2.5 hours and beta ray emission to $Sc^{49}$ which is also radioactive, decaying by beta ray emission to the stable nucleus $Ti^{49}$ (32). It is obvious that radioactive isotopes which are members of a chain cannot be used as radioactive tracers unless the member of interest in the chain is very long-lived with respect to all other members. No cases of importance to biochemists occur in which there is not available another isotope of the same element which does not suffer from this restriction. Thus, calcium possesses in addition to the isotope $Ca^{49}$, the radioactive isotopes $Ca^{41}$ and $Ca^{45}$, either of which can be used as tracers.

The use of nuclear isomers is hedged about with restrictions which depend on the details of the nuclear disintegration. Thus, if one isomer decays into another the resultant isomer may be ejected from the chemical combination in which it is synthesized because the recoil energy available from the departing gamma ray may be sufficient to break the chemical bond. This is true particularly if the gamma ray is "converted," i.e., ejects an extra nuclear electron (as in $Br^{80}$) because in such a case the available recoil energy is an order of magnitude higher. Furthermore, the rearrangement of electronic structure resulting from the loss of an orbital electron in an inner shell is almost certain to lead to disruption of the chemical bond. Consequently if $Br^{80}$ is used to label phenylbromide, the 18-minute isomer will be found invariably dissociated from the phenyl group. Under these circumstances only the 4.5 hour parent isomer is satisfactory as a label. In following such a tracer only measurements on samples exhibiting a 4.5 hour half-life are valid. In the case of the cobalt isomers cited above, there is obviously no difficulty since the product isomer is so long-lived that merely a short aging period to remove all parent isomer is required to obtain a homogenous tracer. It is apparent that general statements on the use of isomeric nuclei as tracers are not feasible. Information on the exact disintegration relations of nuclear isomers is still scanty. It is advisable to avoid the use of isomers the disintegration schemes of which are not known in detail. Fortunately, isomeric nuclei occur only in elements of secondary importance to biochemists, no isomeric nuclei being known for atomic numbers less than twenty.

*Labeling procedures.*—In this section there will be discussed

procedures for the incorporation of isotopic labels in carbon compounds only. The novel dual requirement of maximal yield with minimal amount of reagent introduces rather stringent conditions on syntheses employed in labeling procedures, particularly for an isotope such as $C^{14}$. Other desiderata are maximal recovery of unreacted isotopic reagent and definitive placement of labeling isotope. For short-lived isotopes such as $C^{11}$ the most important requirement is rapidity of synthesis.

Most tracer researches to date have been prosecuted using relatively simple labeled molecules such as carbon dioxide, methane, carbon monoxide, low molecular weight acids, ketone bodies etc. Since isotopic carbon is supplied either as carbonate or cyanide it is necessary to perform preliminary syntheses to bring such material into a form suited for synthesis of complex molecules. A few of these syntheses which have been adapted successfully for labeling purposes will be reviewed.[3]

Of the various reactions reported involving carbon dioxide by far the most extensively employed has been the Grignard reaction for the production of carboxyl-labeled acids. Carboxyl labeled acetic (33), propionic (34, 35), butyric (36), caproic (36, 37), and benzoic acids (38) have been synthesized in this fashion. The reaction is well adapted to the use of small quantities of carbonate.

Starting with isotopic cyanide, a number of useful syntheses leading to carboxyl-labeled acids have been reported. Thus, the reaction between methyl sulfate and labeled cyanide has been used to prepare carboxyl-labeled acetic acid (39). The reaction of labeled cyanide with acetaldehyde has been employed to make carboxyl-labeled lactic acid (35). If doubly-labeled acetaldehyde ($H_3C^*$-$C^*HO$) is used with unlabeled cyanide, $\alpha$-$\beta$ labeled lactic acid results (40). Reaction of labeled cyanide with $\beta$-chlorethanol can be used to make carboxyl-labeled $\beta$-hydroxy propionic acid (34, 35). Succinic acid labeled in both carboxyl positions is easily obtained by the reaction of labeled cyanide with ethylene dichloride (13). From this preparation fumaric acid labeled in both carboxyl positions can be obtained by enzymic dehydrogenation (13). The

[3] Details of methods for synthesis of carboxyl-labeled acetate, carbonyl and/or carboxyl-labeled acetoactate, carboxyl-labeled glycine, carboxyl-labeled pyruvate and $\alpha,\beta$-labeled pyruvate, as worked out by W. Sakami, W. E. Evans, and S. Gurin, are available in a mimeographed pamphlet distributed by the Isotopes Branch, Research Division, Manhattan District, Oak Ridge, Tennessee.

conversion of carbonate to cyanide is accomplished best by reduction at an elevated temperature with potassium in the presence of ammonia (41).

To prepare symmetrically labeled acetylene from labeled carbonate, barium carbonate is reduced to carbide with magnesium in a stream of hydrogen, the carbide then being decomposed to acetylene with water (41). From such acetylene, $\alpha$, $\beta$-labeled acetaldehyde can be made by hydrolysis (40). Preparation of symmetrically labeled oxalic acid from labeled carbonate is accomplished by thermal reduction with potassium (42). From this oxalic acid, labeled formic acid can be obtained by thermal decomposition *in vacuo* at $\sim$180°C. (43).

A number of biosyntheses which can be adapted singularly well to isotope syntheses using small amounts of labeled carbonate have been reported for formic acid (44), doubly labeled acetic acid (45), carboxyl-labeled propionic and succinic acids (46), butyric and caproic acids labeled both in carboxyl and along the chain (37), and methane (47).

Under some circumstances it appears possible to introduce labeled carboxyl or nitrile into organic molecules by direct exchange. Thus, unlabeled $\beta$-hydroxy propionitrile mixed with labeled cyanide at 100° C. for 45 minutes is reported to reach a concentration of labeled carbon in the nitrile group corresponding to 3 per cent exchange (48). Another process involves the rapid exchange at room temperature of labeled carboxyl in acetate with carbon of acetic anhydride (49). Such exchange reactions have not been studied extensively but it is possible a sufficient number may exist to warrant consideration as a means for facilitating labeling syntheses.

In certain syntheses it is desirable to use labeled carbon monoxide as a starting material. The thermal reduction of carbon dioxide by passage over hot metals such as aluminum, zinc, or iron is a familiar method. However, it should be noted that exchange reaction between carbon monoxide and carbon dioxide proceeds with appreciable velocity at $\sim$900° C., practically complete exchange being observed in a few hours if quartz, gold or silver surface catalysts are employed (50). Such a reaction is very attractive for the production of labeled carbon monoxide because complete recovery of both reagents is simple. A method which is claimed to give quantitative conversion of carbonate to carbon

monoxide is based on the reduction of diphenyl carbonate with sodium in warm xylene solution (51).

Labeled carbon monoxide can be converted by a number of simple syntheses to a variety of useful compounds, only a few of which need be mentioned. Particularly attractive procedures from the standpoint of simplicity, minimal quantity of reagent, speed and recovery of isotopic material are based on photosensitized reduction with hydrogen to formaldehyde, formic acid, and a variety of other products (52, 53, 54). Another useful labeling reagent is carbonyl chloride. This compound can be made easily in quantitative yield by means of the classical photochemical reaction between labeled carbon monoxide and chlorine.

It is evident that the introduction of labeled carbon into molecules of complexity corresponding to sterols, carcinogens, higher poly-acids, complex fats, lipids, etc., will not be too easily accomplished in general, concomitantly with satisfying tracer requirements for syntheses involving minimal dilution and loss. Serious delays may result in an otherwise steady expansion of tracer studies. It is appropriate, therefore, to direct attention to the use of hydrogen isotopes as auxiliary carbon labels under conditions where carbon isotopes are not required directly.

A large fraction of biochemical research into the metabolism of carbon compounds has been accomplished with labeling procedures employing deuterium. These researches associated particularly with the name of Schoenheimer (56) may serve as a basis for discussion of future experimentation with deuterium and tritium.

The methods available for preparation of organic deuterium compounds are well known and will be reviewed only briefly. Three general types of synthetic procedures are employed. In the first type deuterium oxide or deuterium is added to an unsaturated compound, or hydrolysis is carried out in the former. Such syntheses are advantageous because the position of the label is known with some certainty. Characteristic of the addition and hydrolysis reactions are the preparation of deuterio-acetylene ($C_2D_2$) by the reaction of calcium carbide with deuterium oxide (57), the reduction of dideuterio-acetylene with chromous chloride to form dideuterio-ethylene (57), reduction of ethylene with deuterium to form deuterio-ethane (58) the synthesis of deuterio-acetaldehyde ($CD_3$ CDO) from deuterio-acetylene in a deuterium oxide solution of phosphoric acid in the presence of mercuric sulphate (59), the

preparation of sodium butyrate-$\alpha$, $\beta$-$d_2$ from the hydrogenation of ethyl crotonate with deuterium (60), synthesis of succinate-$\alpha$, $\beta-d_2$ by hydrolysis with deuterium oxide of ethane-$\alpha$, $\alpha$-$\beta$, $\beta$-tetracarboxylic acid-tetraethyl ester (61), and synthesis of hexadeuterio-benzene by decarboxylation of calcium mellitate with calcium deuterioxide (62, 63). It may be noted that the last mentioned procedure for the deuterio-benzene preparation can be carried out to make the mono-, di-, tri-, as well as hexadeuterio derivations by heating the corresponding carboxylic acids with calcium deuteroxide. Condensation and polymerization reactions involving simple deuterio compounds should be included as a special kind of addition synthesis. Examples are the polymerization of deuterio-acetylene to form hexadeuterio-benzene (64) and the condensation of deuterio-butenyl chloride with glycerol to form tributyrin (65).

The second type of preparation involves exchange with, or substitution of, deuterium with other atoms such as protium, halogens etc. Examples of this sort of reaction are numerous and well known. Substitution by exchange generally results in uncertainty as to exact point of labeling so that suitable degradation procedures must be devised to ascertain in what manner labeling has occurred. In general, exchange occurs in solution at ordinary temperatures wherever direct ionization of hydrogen is possible or wherever mechanisms are available (such as enolization) which can result in ionization. Some exchange occurs even in atomic groupings not ordinarily suspected of exhibiting enolization, such as anions of carboxylic acids (66). A catalyst such as activated platinum is usually required.

In setting up exchange experiments for the purpose of labeling complex compounds the previous experience available in the extensive literature of deuterium can serve as a guide, but no guarantee exists that recourse to the empirical approach will not be necessary. To cite an example, when it was required to synthesize stably-bound deuterium into cholesterol, direct exchange was tried heating suspensions of cholesterol in deuterium oxide in the presence of active platinum. No exchange occurred even at 200°C. (67). The proper conditions were finally ascertained to involve platinum catalyzed exchange in the presence of acetic acid (67). On the other hand acetic acid does not expedite the platinum catalyzed exchange between *meso*-inositol and deuterium oxide (68).

## TABLE I

### Partial Summary of Deuterium—Labeling Procedures

| Method | Reactants | Product | Typical End Products and References |
|---|---|---|---|
| Hydrolysis | Calcium carbide and $D_2O$ | Dideuterio-acetylene | Via dideuterio-ethylene, benzylthioethyl bromide, and benzylhomocysteine, to homocystine-$d_4$, or methionine-$d_3$ (57) |
| Catalytic hydrogenation | Cholestenone and $D_2$ | Coprostanone (4,5-$d_2$) | (71) |
| Catalytic hydrogenation | $\alpha$-keto acids (i.e. pyruvic, $\alpha$-keto glutaric), ammonia and $D_2$ | Deuterio-amino acids | $\alpha$, $\beta$-labeled alanine (72) $\alpha$, $\beta$, $\gamma$-labeled glutamic acid (73) |
| Catalytic hydrogenation | $\alpha$-pyridone $+D_2$ | $\alpha$-piperidone | Via $\delta$-amino valeric acid and $\alpha$-bromo-$\delta$-amino acid to ornithine-$\alpha$, $\beta$, $\gamma$, $\delta$ labeled (65); via dichloro piperidone, $\alpha$, $\alpha$-dichloro-$\delta$-amino valeric acid to proline-$\beta$, $\gamma$, $\delta$-labeled (74) |
| Catalytic hydrogenation | Phenol $+D_2$ | Deuterio-cyclohexanone | Via cyclohexanone oxime, benzoyl-$\epsilon$-amino caproic acid, the $\alpha$-bromo derivative and the potassium phthalimide reaction to lysine-$\beta$, $\gamma$, $\delta$-labeled (75) |
| Catalytic hydrogenation | Diethyl acetal of isobutenaldehyde or isopentenaldehyde $+D_2$ | Diethyl acetal of isobutanal-$\alpha$, $\beta$-$d_2$* or isopentanal-$\alpha$, $\beta$-$d_2$ | Via amino nitrile reaction to valine-$\beta$, $\gamma$-$d_2$ or leucine-$\beta$ $\gamma$-$d_2$ (76) |
| Catalytic hydrogenation | Unsat. fatty acid or ester (i.e. ethyl crotonate) $+D_2$ | Fatty acid (i.e. butyric acid-$\alpha$, $\beta$-$d_2$) | (77) |
| Addition of $D_2O$ | Carbon suboxide ($C_3O_2$) $+D_2O$ | Trideuterio acetic acid | (78) |
| Deuterio-methylation | Methylation of substituted amine (N-$p$ toluene sulfonyl amino ethanol) with deuterio-methyl iodide | Monomethyl-$d_3$-amino ethanol | (79) |
| Deuterio-methylation | Methylation of amine (amino ethanol) with deuterio-formic acid and formaldehyde | Dimethyl-$d_6$-amino ethanol | (79, 80) |
| Deuterio-methylation | Direct methylation of ethanolamine with deuterio-methyl iodide | Trimethyl-$d_9$-amino ethanol (halide) | (81) |
| Deuterio-methylation | Methylation of amino-S-acid (homocysteine) with deuterio-methyl iodide | Methionine (methyl-$d_3$) | (81) |
| Exchange in $D_2O$ | Methylnitrite and $D_2O$ | Trideuterio methylnitrite | (69, 70) |
| Exchange in $D_2O$ | Fatty acid salt and $D_2O$ (activated Pt), i.e. isocaproate and isovalerate | Stably $d$-labeled fatty acids (isocaproic or isovaleric) | Via $\alpha$-bromo acid and ammonolysis to stably $d$-labeled leucine or valine (82) |
| Ecxhange in $D_2O$ | Exchange and rearrangement, i.e. $\Delta^5$ cholestenone in alk.-alcohol $D_2O$ | $\Delta^4$-cholestenone | (83) |

* $d_1$, $d_2$, $d_3$, $d_4$, etc.: these symbols indicate the number of deuterium atoms in the labeled product.

An interesting exchange reaction which should be mentioned in connection with the synthesis of methylated amines is the exchange between methyl nitrite and deuterium oxide which proceeds because of the reversible enolization equilibrium, $CH_3NO_2 \rightleftarrows CH_2:N(OH):O$. The trideuterio methyl nitrite can be obtained easily by exchange in acetate buffer at 80° C. (69, 70). After reduction with iron filings in hydrochloric acid the benzoyl derivative is prepared and heated with the phosphorus pentahalide to obtain the corresponding trideuterio methylhalide (70).

A partial summary of the two kinds of synthetic procedures discussed to this point is presented in Table I.

The third type of synthesis exploits the production of labeled compounds by organisms grown in deuterium oxide, or on deuterium-containing substrates. This method although highly advantageous because of its flexibility as a source of an endless variety of metabolites suffers from two serious drawbacks: first, the low isotopic content occasioned by the initial low deuterium level in the circulating plasma and fluids coupled with dilution by endogenous reactions; secondly, necessity for degradative analytical procedures to define the position of the label. Nevertheless biosynthesis may be used to advantage under conditions in which the initial isotopic content can be raised to relatively high levels, as in the application of tritium. This point will be discussed at more length below.

Experiments with carbon chains labeled by carbon-hydrogen linkages are subject to limitations which have already been discussed and will be recalled briefly. Variations in stability of carbon-deuterium occurs under a variety of conditions, some carbon-deuterium bonds being completely stable to direct exchange, others such as those found in a few amino acids, i.e., glycine, glutamic acid, cystine and tyrosine, containing slowly exchangeable hydrogen (84). The ambiguities arising from the indirect nature of the label have been mentioned for one case previously in the text and obviously constitute a general, more serious kind of limitation.

Despite considerations of this nature there is a formidable literature to bear witness to the great versatility of deuterium as an auxiliary carbon tracer (19, 56). An equal versatility for tritium has not been demonstrated as yet owing to the small supply of tritium available hitherto. It is to be expected, however, that all labeling procedures applicable to deuterium are applicable with

little change to tritium. Whenever deuterium and tritium have been studied together it has been found that the two isotopes parallel each other closely in exchange characteristics (85). Tritium possesses advantages over deuterium as a tracer stemming from its great dilution range. From present sources samples of tritium water in quantities of 5 to 10 c.c. are available with a total radioactivity of $10^{10}$ to $10^{11}$ counts per min., as assayed with a Geiger-Muller tube. One c.c. of such a sample as hydrogen gas can be introduced into the counting tube so that if 100 counts per min. is taken as the lower limit for precision counting (1 to 5 per cent error in 30 minutes of counting) then dilution ratios of $10^7$ to $10^8$ are available.

With such dilutions, many types of syntheses denied with deuterium become available with tritium. As an example, histidine prepared by biosynthesis in the mouse assays only 0.24 atom per cent deuterium beginning with a deuterium level in the diet of 1.50 to 1.60 atom per cent (86). Such histidine can be diluted only about twenty fold for precision density assay. It can be calculated that biosynthesis in tritium water of the isotopic composition available at present would yield histidine-t which could be diluted 500 to 1000-fold while still permitting precision assay. Similar considerations apply to the other amino acids obtainable by biosynthesis from isotopic water. Considerably stronger samples of tritium are in prospect from the uranium pile reactor so that samples some orders of magnitude more concentrated than those now possible should be available by this technique. Radiation dosage tolerance for tritium should be considerably higher than those quoted for other radioactive isotopes because of the very soft radiation emitted (87).

Spectacular increases in dilution factors obtainable should attend the use of tritium in chemical syntheses because radiation damage would not be a limiting factor. Thus, it has been shown that cholesterol containing 5.70 atom per cent deuterium can be obtained by exchange catalyzed with acetic acid in 88 per cent deuterium water (67). In this case the deuterium assay would permit a dilution of many hundredfold. Using tritium water with an initial specific activity of $10^{12}$ counts per min. per mole of hydrogen, one should be able to obtain triturated cholesterol which would withstand dilutions up to $10^6$ with resultant precision of better than 5 per cent. The importance of such considerations for

the general synthesis problem is evident. Carcinogens, such as methyl cholanthrene, could be labeled in a manner analogous to that discussed for cholesterol. The labeling of such a compound with tracer carbon rather than tracer hydrogen might require a synthesis which would be either wasteful or impossible. In all cases where tritium labeling is feasible it should provide greater sensitivity than the carbon isotopes because of the very high specific isotope content available with tritium.

It can be concluded that the potentialities of tritium as an auxiliary tracer for carbon are such that a considerable extension in carbon tracer research can be expected from its exploitation. The prospect of such exploitation is nebulous at present because no large supply of tritium is available. A distinct disadvantage of tritium labeling techniques are the tedious and difficult assay procedures required at present (88). A promising development in the improvement and simplification of assay procedures for tritium has recently appeared in the description of a modified Lauritsen electroscope for precision assay of tritium as well as $C^{14}$ (89).

Researches on the fate of substrate molecules in metabolism can be extended by the use of multiple labeling techniques, a development pioneered by Schoenheimer and his collaborators. An early example is the use of leucine and lysine labeled with deuterium on the carbon chain and $N^{15}$ in the amino groups to demonstrate that although both acids are indispensable nutritional factors, leucine undergoes partial resynthesis in the animal organism by removal and replacement of the amino group while lysine once deaminated is not resynthesized (75).

While syntheses employed in double labeling are in no way unique, the methods used in two cases may be cited to elaborate further on the material presented in Table I. *dl*-Leucine, doubly labeled, can be prepared by treatment of deuterio-isocaproic acid, conversion to the ethyl-$\alpha$-bromo ester and amination by $N^{15}$ labeled potassium phthalimide (90). Resolution of the racemic mixture is accomplished by means of the brucine salt of the formyl derivative (91). For the synthesis of doubly-labeled *dl*-lysine, deuterio cyclohexanone is prepared by catalytic hydrogenation of phenol followed by conversion with hydroxylamine to the oxime. The oxime rearanges in hot sulfuric acid to deuterio-benzoyl-$\epsilon$-amino caproic acid. The ethyl-*d*-bromo ester of this acid is aminated with $N^{15}$ labeled potassium phthalimide (75). The resultant

lysine contains deuterium in the $\beta$, $\gamma$ and $\delta$ carbons and $N^{15}$ in the $\alpha$-amino group. Resolution is accomplished via the camphorates.

As another example of a double-labeling synthesis, there may be cited the preparation of *dl*-proline containing deuterium and $N^{15}$ (75). $N^{15}$ labeled hydroxynicotinic acid is synthesized by the reaction between $N^{15}H_3$ and the methyl ester of coumalic acid. The hydroxynicotinic acid is then decarboxylated to $\alpha$-pyridone which is hydrogenated catalytically with deuterium to deuterio-$\alpha$-piperidone, which in turn is converted to the corresponding $\beta$-dichloropiperidone. Acid hydrolysis to $\alpha$, $\alpha$-dichloro-$\delta$-aminovaleric acid followed by reduction with sodium amalgam to proline completes the synthesis, the product being labeled with deuterium in the $\beta$, $\gamma$, and $\delta$ positions of the pyrrolidine ring.

The application of the double-label techniques can be exemplified by reference to researches concerned with mechanisms for the conversion of methionine to cystine in the animal organism. In early phases of this work, $S^{35}$-labeled methionine was employed and it was found that in rats fed such methionine, labeled cystine could be isolated from the hair (92). From this result it could be concluded only that methionine sulfur could be used in the formation of cystine. To determine whether the carbon chain of methionine could be used in the formation of cystine it was necessary to label the carbon chain. The use of an isotopic hydrogen label was inadvisable because of the possibility of labilization. It was found, in fact, that when dideuterio-methionine, labeled in the $\alpha$ and $\beta$ positions, was fed, the cystine isolated contained no deuterium (93). The problem was attacked directly (94) by feeding methionine labeled with both $C^{13}$ and $S^{34}$ ($CH_3S^{34}C^{13}H_2C^{13}H_2CHNH_2$-$COOH$). The sulfur label provided a control in that its presence or absence in the final cystine isolated could be used as an index of utilization of methionine sulfur. Consequently it could be ascertained to what extent the methionine carbon was utilized under conditions known to result in incorporation of methionine sulfur. When doubly-labeled methionine was fed to rats, the cystine isolated from hair contained sulfur, 80 per cent of which had been derived from methionine, but no significant amount of the original methionine carbon. It followed that the carbon chain of methionine was not a part of any of the precursors in the formation of cystine *in vivo*. It appears actually that serine is such a precursor (95, 96).

These researches also illustrate how normal synthetic procedures may require modification before application to tracer studies. Thus, in the original synthesis of $S^{35}$-labeled methionine, a synthesis was employed via benzylmercaptan, through a malonic ester synthesis to benzylhomocystine (97). It was found necessary to modify this synthesis because undue loss in yield occurred in a step involving conversion of benzylthioethyl malonic ester to benzylhomocysteine. Replacement of malonate by phthalimidomalonate was found to result in a more satisfactory procedure (92). This synthesis still remained inconvenient when it was required that small amounts of labeled methionine be available at intervals. A method was substituted based on synthesis via $\alpha$-benzamido-$\gamma$ chlorobutyric acid ethyl ester (98). In the work with the doubly-labeled methionine it was necessary to evolve a procedure for continuous circulation of ethylene dichloride used in one of the steps of the synthesis because the small amounts employed made direct recovery impractical (94).

Intriguing possibilities latent in multiple labeling procedures are enhanced by the availability of a number of isotopic labels for each element. Thus, these are the isotopic pairs $H^2$-$H^3$, $C^{13}$-$C^{14}$, $Na^{22}$-$Na^{24}$, $S^{34}$-$S^{35}$, $Ca^{41}$-$Ca^{45}$, $Br^{80}$-$Br^{82}$, $I^{130}$-$I^{131}$, etc. It is apparent that future extension of tracer techniques will involve much exploitation of multiple labels.

In conclusion a type of labeling which has received little attention may be mentioned. It is possible to exploit the fact that the equilibrium constants of many exchange reactions involving isotopic species deviate markedly from unity (99). An example of such an equilibrium important in geochemical considerations is the reaction,

$$\underset{\text{(gas)}}{CO_2^{16}} + \underset{\text{(liq.)}}{H_2O^{18}} \rightleftarrows \underset{\text{(gas)}}{CO_2^{18}} + \underset{\text{(liq.)}}{H_2O^{16}}$$

for which the calculated values of the equilibrium constant at 0° and 25°C. are 1.097 and 1.080 respectively. These values have been checked experimentally (100). The manner in which such exchange equilibria can be exploited is illustrated again by reference to researches into the mechanisms operative in photosynthesis, in this case work designed to clarify the role of water as the ultimate source of photosynthetic oxygen (101).

Green algae were allowed to photosynthesize in a buffer solu-

tion (pH$\sim$10). In some experiments the water was labeled with $O^{18}$, in others the carbonate was labeled. In both cases the $O^{18}$ content of the evolved oxygen was equal to that of the water within the limits of precision of the mass spectrometer assay, and differed widely from that of the carbon dioxide. A slow exchange of $O^{18}$ between carbon dioxide and water was observed to occur in accordance with the reaction mentioned above. This rate of exchange was negligible in comparison with the rate of oxygen evolution, so it was concluded that the oxygen originated solely from the water.

Such a conclusion depended on the assumption that the isotope exchange was no more rapid in the cells, and especially in the chloroplasts where oxygen evolution actually occurs, than in the outside medium where the exchange was measured. However the rate of exchange is known to increase rapidly with decreasing pH. At pH $<6$, the randomization of $O^{18}$ could be calculated to be rapid enough (102) to invalidate the conclusion that carbon dioxide is excluded as a source of oxygen. It is known that in some plants the vacuolar pH can be maintained as low as 4.5 despite wide variations of pH in the external medium.

The difficulties resulting from randomization of $O^{18}$ can be avoided to a large extent by making use of the small difference between $O^{18}$ contents of water and carbon dioxide in equilibrium to trace the origin of photosynthetic oxygen. It is necessary in this type of application to resort to the very sensitive density assay methods based on the use of the submerged float (3, 103, 104). When algae are allowed to photosynthesize in ordinary water equilibrated with carbon dioxide by means of beef carbonic anhydrase, the $O^{18}$ content of the envolved oxygen is found to be nearly identical with that of the water and significantly lower than that of the carbon dioxide (105). The density of water prepared from the oxygen of equilibriated carbon dioxide is some 8 to 10$\gamma$ units higher than the density of water in equilibrium with carbon dioxide. The oxygen evolved photosynthetically yields water with a density within 1$\gamma$ unit of the density of water. There remains the possibility that evolved oxygen may arise from carbon dioxide but equilibrate with water through the reaction

$$\underset{\text{(gas)}}{O_2^{16}} + \underset{\text{(liq.)}}{H_2O^{18}} \rightleftarrows \underset{\text{(gas)}}{O_2^{18}} + \underset{\text{(liq.)}}{H_2O^{16}}$$

for which the calculated equilibrium constant at 25° C. is 1.012.

Such oxygen would yield water with density approximately 1 $\gamma$ unit higher than that of water. However the rate of exchange in this reaction is exceedingly slow at all pH values of biological significance and it is very improbable that such an equilibration occurs. It follows therefore that the original investigations employing $O^{18}$ at a high level of concentration are correct and the conclusion that water is the sole source of photosynthetic oxygen is borne out. Similar results have been obtained for the two land plants, sunflower and Coleus.

The extended discussion accorded this type of labeling is warranted by the importance of the experimental result derived. However it is apparent that this "equilibrium" type of tracer is extremely limited in application and cannot be expected to assume general importance in tracer studies.

*Concluding remarks.*—Because of the comprehensive nature of the subject matter, the organization and treatment of the literature has deviated considerably from that appropriate to the usual review article concerned with a highly specialized topic in biochemistry. The justification for the stressing of certain admittedly limited aspects of tracer methodology lies in the impossibility of presenting within the space limitations of these *Reviews* adequate critical commentaries on more crucial aspects such as the characterization of metabolic intermediates, origins of excretory products, reversibility of enzymic syntheses *in vitro* kinetics and dilution calculations for specification of metabolic mechanisms, and analysis by isotopic dilution methods. It is quite certain that, as the use of isotopes in biochemical research expands, integrated discussions of such aspects will become feasible and desirable.

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# THE CHEMISTRY OF THE STEROIDS

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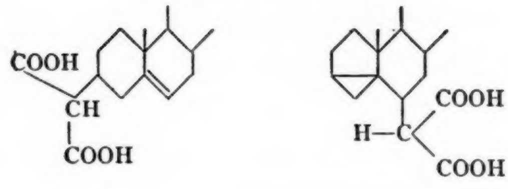
In this review the authors have attempted to cover all essential publications on the chemistry of the natural sterols, bile acids, and steroid hormones since the review of 1945, with the exception of (*a*) the specialized material covered in the article published in Volume XV (1946); (*b*) a few articles of 1945 because of lack of space; (*c*) structural determinations in the fields of the cardiac aglucones, steroid alkaloids, toad poisons, and sapogenins; and (*d*) attempts at total synthesis of steroid compounds.

*Sterol reactions.*—A new method for the preparation of 7-dehydrocholesterol has been described (1). It has been found that when cholesteryl acetate is treated with N-bromo-succinimide bromine enters the sterol nucleus at position 7. On treatment of this latter compound with dimethyl aniline, 7-dehydrocholesterol is produced.

A stable ozonide of cholesterol has been reported (2). Ozonolysis, however, proceeds past the theoretical amount if carried out in certain solvents. The unusual stability of this ozonide is demonstrated by the fact that it can be chromatographed, and that it does not decompose on standing at room temperature. In solution it is apparently dimeric.

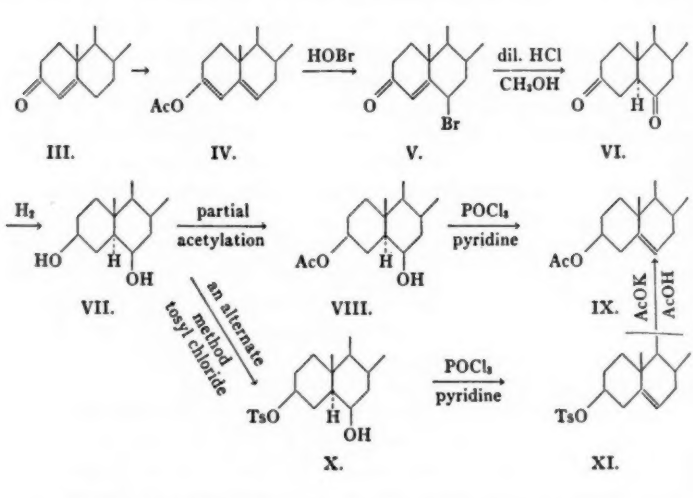
A novel method for the preparation of sterol dichlorides has been described (3). With sterols, and sterol esters unsaturated at the $C_5$—$C_6$ position iodobenzene dichloride gives two isomeric dichloro-sterols and sterol esters. Although the steric configurations of the halogens have not been determined it has been established that the higher melting isomer of the sterol ester on hydrolysis gives the normal known sterol dichloride.

The action of sodiomalonic ester on cholesteryl *p*-toluenesulfonate has been studied (4), and two isomeric substituted malonic acids have been obtained. Evidence has been presented which indicates that one of them is the *i*-isomer, and that the following structures can be assigned to these two isomeric forms.



I. II.

An interesting method for the conversion of a $\Delta^4$-3-keto steroid into a $\Delta^5$-3-hydroxy steroid has been discovered (5). The following reaction scheme briefly outlines how this is accomplished, and although the separate steps are many the yields appear to be good.



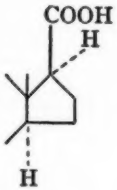

The method of molecular rotation differences frequently employed in recent years to establish structures of steroids has been extended (6), and it has been found that triterpenoids can be distinguished readily from sterols. Evidence is submitted to show that $\alpha$-spinasterol, episterol, ascosterol, and faecosterol are incorrectly formulated in the literature.

Further studies (7) on the dehydrohalogenation of 3-chlorocholestanone-6, and 3-chloroandrostandione-6, 17 have been reported, and it is now quite certain that the unsaturated ketones so produced are actually $\Delta^2$ isomers and not $\Delta^4$ compounds as previously described.

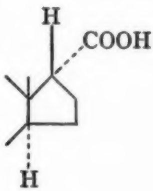
In concluding this section of the review the authors would mention the recent studies (8) on the microbiological oxidation of 3-hydroxy steroids to the corresponding keto derivatives by certain species of Proactinomyces. Oxidation by these organisms appears to be a general reaction, and is independent of the side chain at position 17, for stigmasterol, coprosterol, testosterone, and 17-hydroxy steroids are susceptible.

*The bile acids* (*stereochemistry*).—The action of methanolic sodium methoxide on certain normal *etio* acid methyl esters has been investigated (9). Results indicate extensive isomerization at the $C_{17}$ position. This effect is reversible. Thus, by this method 3($\beta$)-hydroxy-17-*isoetioallo*cholanic acid, and 3($\beta$)-hydroxy-17-*isoetio*cholanic acid were isomerized into the equilibrium mixture of both forms and the two products separated and isolated in a pure state.

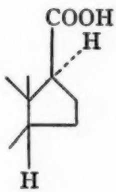
By means of a study of space models certain conclusions have been drawn (10) as to the stereochemistry of the four *etio* acids isomeric at $C_{14}$ and $C_{17}$. (See page 677.)



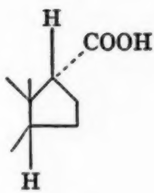

XII.




XIII.



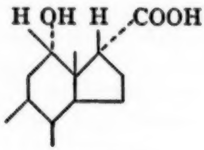

XIV.



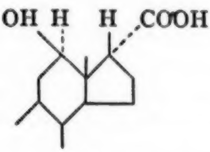

XV.

The acid which has the configuration XIII should exhibit the greatest hindrance. Experimentally the ester of the *etio* acid with the *iso* ($\alpha$), configuration of the carboxyl group, prepared by the sodium methoxide inversion of the normal ester, and the normal *trans* relationship of rings C and D showed the slowest

rate of saponification. This fact supports the formulation of the side chain as $\beta$. The results of other investigators (11, 12) are of interest in this connection. It is known that all naturally occurring steroids have the same configuration of the side chain. Final evidence has now been submitted in the case of desoxycholic acid to



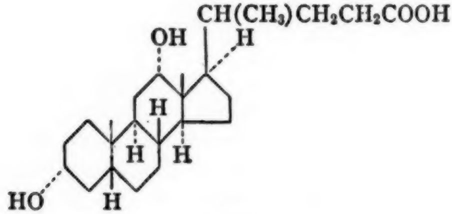

XVI.




XVII.

show that the side chain has a $\beta$ configuration, and that the $C_{12}$ hydroxyl group has an $\alpha$ configuration. The four *etio* acids epimeric at $C_{12}$ and $C_{17}$ have been prepared. Lactone formation of a 12-hydroxy *etio* acid is possible only when the hydroxyl and carboxyl groups lie on the same side of the ring system, and also when they both lie on the side opposite the $C_{13}$ methyl group, which is known to be $\beta$ configuration. Experimentally it was found that only one of the four isomeric hydroxy acids gave a lactone. Therefore, it must be concluded that this one is of the configuration XVI and not XVII.

Since this acid has the natural configuration of the hydroxyl group, that is, the same as in desoxycholic acid and since the configuration of the carboxyl group is *iso* because of the fact that it was prepared by inversion of the normal acid, desoxycholic acid must have the following structure (XVIII).

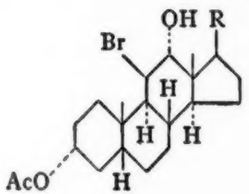



XVIII.

*The bile acids* (*reactions of Ring C*).—A method for the introduction of a $\Delta^{11}$ double bond into the steroid nucleus has been described (13). Although this work has been published only recently it is evident that it actually precedes the methods of other investigators. In this method methyl 3($\alpha$)-hydroxy-12($\alpha$)-benzoxycholanate was pyrolyzed at 315°C. to give methyl $\Delta^{11}$-3($\alpha$)-hydroxycholenate. The position of the double bond was established by oxidation to choloidanic acid, and by reduction of the $\alpha$-oxido compound to desoxycholic acid.

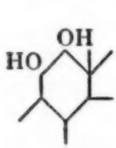
These 11–12 oxides have interesting properties (14, 15, 16). On treatment with hydrogen bromide a bromohydrin (XIX) is produced. Oxidation of this latter compound gives one of two possible bromoketones, both of which can be prepared by direct bromination of the corresponding 12-keto derivative. The bromoketone prepared from the bromohydrin readily loses hydrogen bromide to give the $\Delta^{9,11}$-12-keto derivative. The other possible bromoketone does not easily lose hydrogen bromide. These facts permit certain stereochemical formulations. Since opening of the oxide ring is probably accompanied by inversion, and since reduction of the oxide compound gives desoxycholic acid, the $C_{12}$ hydroxyl in the bromohydrin has the same configuration as in desoxycholic acid, and the $C_{11}$ bromine is of the opposite configuration. Therefore, if we assume *trans* elimination of hydrogen bromide the $C_{11}$ bromine must be $\beta$, since the $C_9$ hydrogen is almost certainly $\alpha$, that is, *trans* to the $C_{10}$ methyl group, the ultimate point of reference. Hence the bromohydrin (XIX) is an 11($\beta$)-bromo-12($\alpha$)-hydroxy compound. Again this shows the $C_{12}$ hydroxyl group in desoxycholic acid to be $\alpha$ configuration.

It is also of interest to record that 11, 12 oxido compounds react

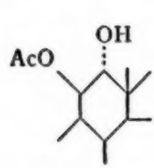



XIX.

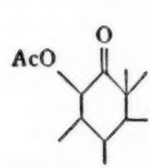
with acetic acid and mineral acids to give three identifiable products (15). Thus, the particular oxide under discussion gives methyl $\Delta^{9,11}$-3($\alpha$), 12-diacetoxycholenate, methyl 3($\alpha$)-acetoxy-11, 12-dihydroxycholanate (XX) and methyl, 3($\alpha$), 11($\beta$)-diacetoxy-12-($\alpha$)-hydroxycholanate (XXI). The configuration of the last compound is assigned by analogy with the bromohydrin (XIX). Oxidation of XXI (amorphous) gives the acetoxy ketone (XXII). In this connection it should also be noted that potassium permanganate in acetic acid acts on methyl $\Delta^{11}$-3($\alpha$)-acetoxycholenate to give the same oxide described above (16), which with anhydrous hydrogen fluoride at $-80°$C. gives a crystalline glycol evidently different from XX.




XX.

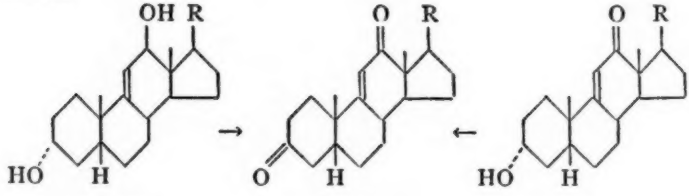



XXI.




XXII.

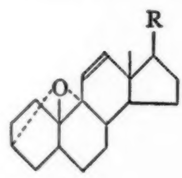
Certain other reactions of $\Delta^{11}$ compounds should be recorded. Thus, $\Delta^{11}$-3($\alpha$)-hydroxycholenic acid, and several of its derivatives have been found to react with bromine to give dibromides (17, 18, 19). With aqueous alkali these dibromo compounds yield $\Delta^{9,11}$-3($\alpha$), 12-dihydroxycholenic acid or its derivatives. The structure of this latter type of compound (XXIII) was shown by oxidation to a diketo acid (XXIV). This same acid was obtained from the hydroxy keto acid (XXV) previously prepared (20) in 1940. The $C_{12}$ hydroxyl was found to show unusual activity. For example, in methanol containing a trace of mineral acid approximately a quantitative yield of the methyl ether was obtained.




XXIII. XXIV. XXV.

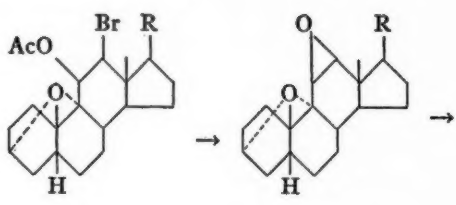
Besides pyrolytic methods there are other ways (21) of preparing $\Delta^{11}$ bile acids and their derivatives. For example, the $C_{12}$ hydroxyl group in desoxycholic acid (XVIII) can be tosylated. Removal of *p*-toluene sulfonic acid can be effected with pyridine at 150°C. In a series of homologues varying from the cholanic acid type to the *etio* acid type the yields of $\Delta^{11}$ steroid increased steadily as the side chain shortened. This is the reverse of the trend shown by the pyrolytic procedure.

The preparation of a novel type of bile acid derivative, 3,9-epoxycholenic acid (XXVI) has been announced (22). The starting material for this preparation is methyl $\Delta^{9,11}$-3($\alpha$)-hydroxy-12-methoxycholenate. With a hydrogen halide the corresponding allyl halide is produced, which in the presence of pyridine or aqueous bicarbonate gives a cyclic oxide of the formula



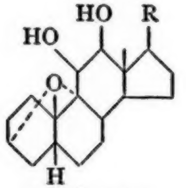
XXVI.

Evidence for this structure is presented. Of special interest is the fact that the compound as prepared contains no hydroxyl groups, since acetic anhydride and chromic acid are without effect. The compound, however, forms a dibromide, and one of the bromine atoms can be replaced by the acetoxy group forming XXVII. This substance with alkali gives a dioxide (XXVIII) which is opened with mineral acid to yield two stereoisomeric oxido glycols (XXIX). Chromic acid oxidation of these latter compounds yields an oxido



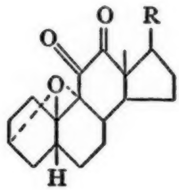
XXVII. XXVIII.

diketone whose absorption spectrum is consistent with the following formulation (XXX).



two isomers

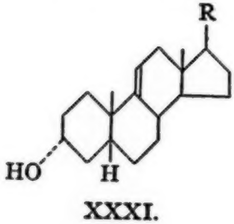
XXIX.



XXX.

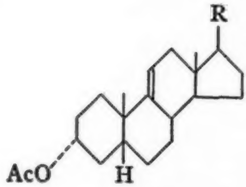
The action of potassium permanganate in pyridine solution on $\Delta^{11}$-cholenic acid has been studied (23), and it has been found that the second theoretically possible *cis* glycol is formed. It is to be recalled that the other *cis* glycol is produced (24) by the action of osmium tetroxide on this acid. $\Delta^{11}$-3-Keto cholenic acid also yields a glycol when treated with potassium permanganate in pyridine solution. Efforts to convert this latter compound to the 11-keto derivative by thermal dehydration were unsuccessful. Traces of the 11-ketone were obtained, however, from the *cis* glycol produced by the action of potassium permanganate on $\Delta^{11}$-cholenic acid.

Improvements in the method of preparation of $\Delta^{9,11}$ steroids have been described (25, 26). From desoxycholic acid yields of $\Delta^{9,11}$-3($\alpha$)-hydroxycholenic acid (XXXI) up to 50 per cent have been obtained. It has also been shown that $\Delta^{9,11}$ steroids can be produced in good yield by the reduction of 11-keto compounds, followed by dehydration at room temperature with phosphorus oxychloride in pyridine. With hypobromous acid methyl 3($\alpha$)-acetoxy-$\Delta^{9,11}$-cholenate (XXXII) has been found to give a bromohydrin (amorphous), from which, after oxidation and reduction,

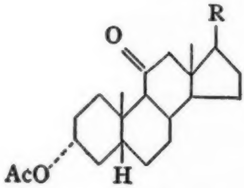


XXXI.

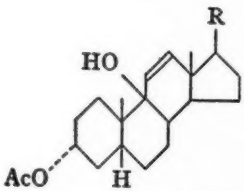
methyl 3($\alpha$)-acetoxy-11-ketocholanate (XXXIII) can be isolated. In acetic acid solution potassium permanganate reacts with XXXII to give two oxides, one of melting point 118°C. identical with that obtained with perbenzoic acid, the other of melting point 146°C. The action of hydrogen fluoride on the lower melting oxide gives rise to two compounds formulated as XXXIV and XXXV respectively.



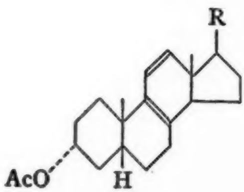

XXXII.




XXXIII.



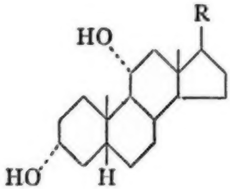

XXXIV.



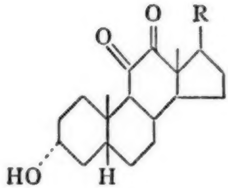

XXXV..

*The bile acids* (*Ring C—two oxygen functions*).—A new method for the introduction of the $C_{11}$ keto group into the steroid nucleus has been described (30). This method involves the replacement of the 12-hydroxy group in 3($\alpha$)-acyloxy-12-hydroxy-11-ketocholanic acid with bromine. The product so obtained is then submitted to reduction followed by hydrolysis. Thus, 3($\alpha$)-hydroxy-11-ketocholanic acid is obtained.

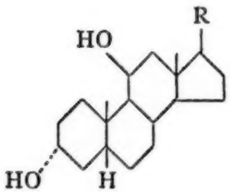
By the Wolff-Kishner reduction of 3($\alpha$),11($\alpha$)-dihydroxy-12-keto*etio*cholanic acid prepared by known methods from *etio*desoxycholic acid, 3($\alpha$),11($\alpha$)-dihydroxy*etio*cholanic acid (XXXVI) has been obtained (27). An 11-hydroxy compound can also be prepared (28) by the action of hydrazine and sodium ethylate on 3($\alpha$)-hydroxy-11,12-diketocholanic acid (XXXVII). In this reaction 3($\alpha$),11($\beta$)-dihydroxycholanic acid (XXXVIII) is produced. The whole series of 3($\alpha$),12-dihydroxy-11-keto acids (XXXIX) has also been prepared (29), in which the side chain, R, is of the *nor-*, *bisnor-*, and *etio*cholanic acid types.



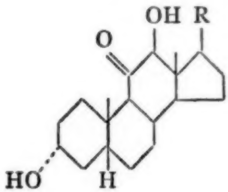

XXXVI.




XXXVII.



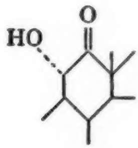

XXXVIII.



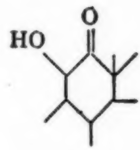

XXXIX.

All past attempts (31, 32) to prepare 11-hydroxy steroids by the Wolff-Kishner reduction of a supposedly 3,11-dihydroxy-12-ketocholanic acid have failed. The reason for this is that in reality

the starting material was 3(α),12(β)-dihydroxy-11-ketocholanic acid (33). However, the preparation of the two stereoisomeric acids, 3(α),11(α) and 3(α),11(β)-dihydroxy-12-ketocholanic acids (XL and XLI) has now been accomplished (34). When methyl 3(α)-acetoxy-12-ketocholanate is brominated, two isomeric 11-bromo-12-keto compounds are formed. Separation of these isomers has been effected, and on treatment with cold alcoholic alkali each gives an 11-hydroxy-12-keto derivative, and each isomer also forms a hydrazone in contrast with the 11-keto-12-hydroxy compounds which do not react with hydrazine.

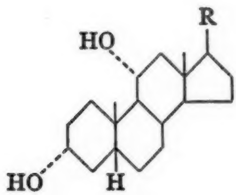


XL.

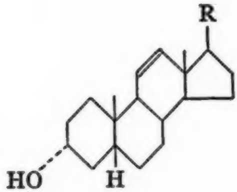


XLI.

The Wolff-Kishner reduction of both XL and XLI has been found (34, 35, 36) to give two main products, XLII and XLIII respectively. In addition, 3(α),11(α),12(α)- and 3(α),11(α),12(β)-trihydroxycholanic acids (XLIV and XLV) have been isolated. By other methods the other two stereoisomeric modifications, XLVI and XLVII, have been prepared. By employing two well-substantiated criteria for the β configuration of an 11-hydroxy group—marked difficulty of esterification and facility of dehydration—and by other correlations, the configurations of all four 3(α),11,12-trihydroxycholanic acids have been determined. The two acids in question isolated from the Wolff-Kishner re-

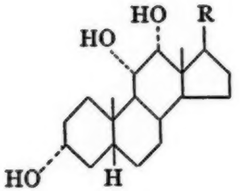


XLII.

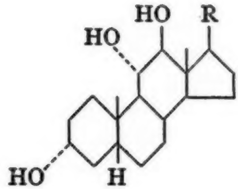


XLIII.

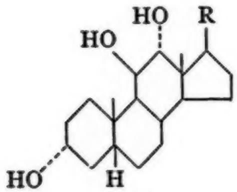
duction of XL and XLI have the structures assigned in XLIV and XLV.



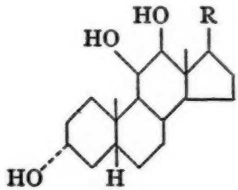
XLIV.



XLV.

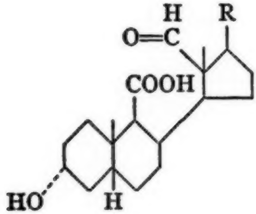


XLVI.

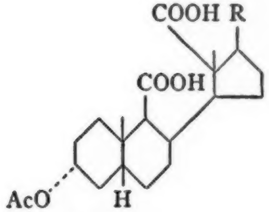


XLVII.

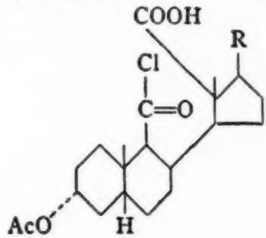
In concluding this section, it should be noted that studies have been made (37) on the nature of the products formed in the periodate oxidation of 3($\alpha$),12($\beta$)-dihydroxy-11-ketocholanic acid. This compound was found to give an aldehyde acid, XLVIII. Further oxidation of the 3-acetoxy derivative gave an acid of structure XLIX. The 3($\alpha$)-acetoxy-24-methylester of XLVIII could be converted into an acid chloride (L) which, however, did not react with diazomethane.



XLVIII.

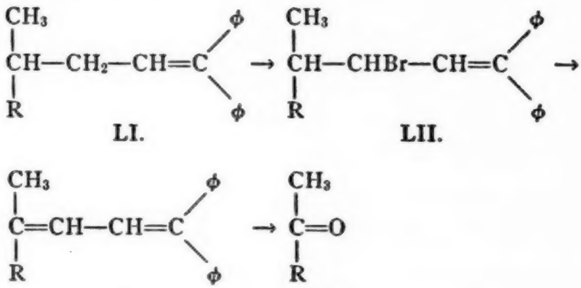


XLIX.



L.

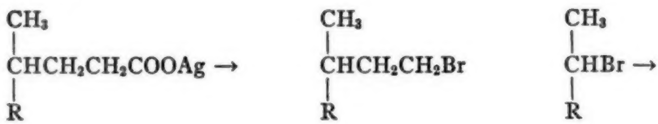
*Bile acids* (*side chain reactions*).—In a recent series of papers (38) a new and efficient method is described for the preparation of 20-ketopregnanes from bile acids. The diphenylethylene derivative (LI), prepared as in the classical Barbier-Wieland procedure, is brominated at the $C_{22}$ position with N-bromosuccinimide. Refluxing of this compound (LII) in carbon tetrachloride solution yields the corresponding diene (LIII). Chromic acid oxidation of this compound gives the 20-ketopregnane (LIV) in a yield of 30 to 40 per cent based on the diphenylethylene (LI). Thus far cholic, desoxycholic, hyodesoxycholic (39), *allo*cholanic, lithocholic, 3($\beta$)-hydroxy*allo*cholanic, and $\Delta^5$-3($\beta$)-hydroxycholenic acids have been successfully degraded.



LI. LII. LIII. LIV.

The successful conversion of pregnanediol-3($\alpha$),12($\alpha$)-one-20 to *etio*cholanediol-3($\alpha$),12($\alpha$)-one-17 has been announced (40). This was accomplished by treatment of the former compound with ethyl nitrite and sodium ethoxide. The intermediate oxime first formed is hydrolyzed with mineral acid.

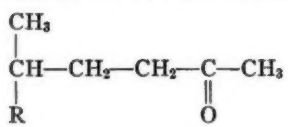
The silver salts of several bile acid derivatives have been shown (41) to react with bromine to form compounds in which the carboxyl group is replaced by a bromine atom (LVI). Although the further degradation of the primary halide so prepared did not seem promising, the secondary halide (LVII) prepared from the *bisnor* acid was found to be readily converted to a pregnene LVIIIa or LVIIIb.



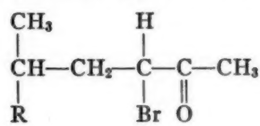
LV. LVI. LVII.

LVIIIa. LVIIIb.

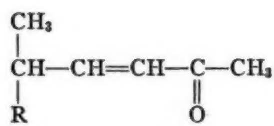
The results of certain other studies on the degradation of the side chain in a bile acid derivative should be noted. Thus, the preparation of a number of phenyl and methyl ketones by the interaction of bile acid chlorides, and the appropriate organic cadmium or zinc aryl or alkyl has been described (42). The acid chloride of cholanic acid, after successive treatment with diazomethane, hydrogen bromide, and zinc has been found (43) to give norcholanyl methyl ketone (LIX). Bromination gives a compound



LIX.

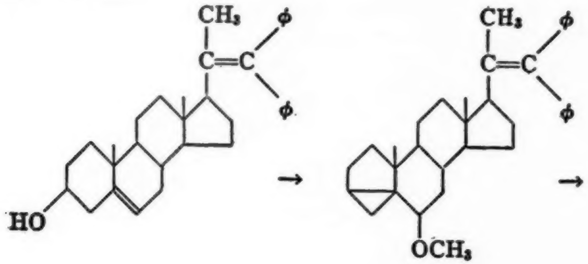


LX.

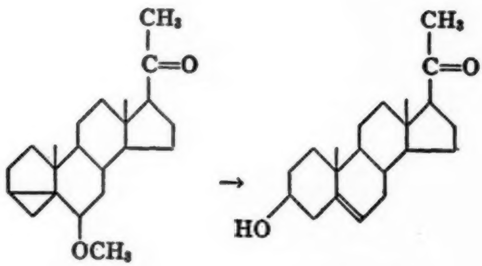


LXI.

of structure LX, which on refluxing with collidine yields an $\alpha,\beta$-unsaturated ketone LXI. Subsequent oxidation, however, afforded only small yields of *bisnor*cholanic acid. Studies of the ozonolysis and rearrangement of certain *i*-ether derivatives, LXII to LXIV, have been made (44). Results indicate that this method does not appear at present to be as efficient as certain other procedures already described in the literature. The stepwise degradation of 3($\alpha$),11($\alpha$)-dihydroxycholanic acid to the corresponding *etio* acid has been accomplished (45). Finally, drastic chromic acid oxidation of methyl 3($\alpha$)-acetoxy-11-keto-12-bromocholanate has been found (46) to yield a small amount of the corresponding 17-ketone.



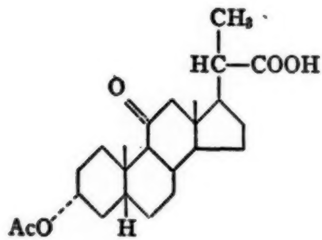
LXII.



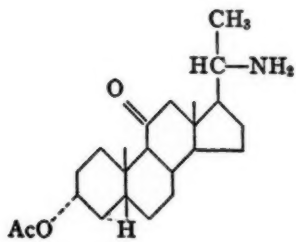
LXIII. LXIV.

*Partial synthesis of Kendall's Compound E.*—Structural studies of previous years on Compound E, one of the physiologically active compounds of the adrenal cortex, have shown it to be pregnene-4-diol-17($\beta$), 21-trione-3,11,20 (LXXVI). The partial synthesis of this compound has now been described (47). The starting

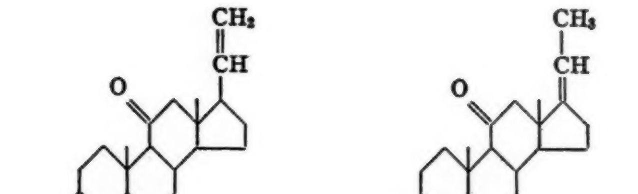
material, 20-amino-3(α)-acetoxy-11-ketopregnane (LXVI), was prepared by a Curtius degradation of the corresponding *bisnor* acid LXV. Diazotization in pyridine gave a mixture of pregnenes, LXVII, from which, after ozonolysis, *etio*cholanol-3(α)-dione-11, 17 (LXVIII) could be separated. Application of the Nef reaction was found to give an ethinyl derivative, LXIX, which on reduction produced pregnene-20-diol-3(α),17(α)-one-11 (LXX). By an allylic rearrangement, pregnene-17-diol-3(α), 21-one-11 (LXXI) was obtained. It was found that this compound in the form of its half ester could be oxidized to pregnene-17-ol-21-dione-3,11 (LXXII). Hydroxylation of this latter compound, followed successively by bromination and treatment with pyridine yielded pregnene-4-triol-17(β),20(β),21-dione,3,11 (LXXIII) identical with the natural substance U of Reichstein. Partial acetylation gave the 21-monoacetate, LXXIV, from which on oxidation with chromic acid adrenosterone (LXXV) and compound E (LXXVI) were obtained.



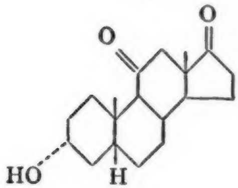

LXV.



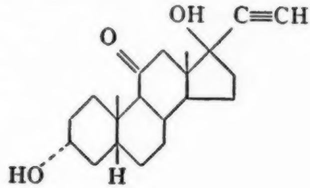

LXVI.



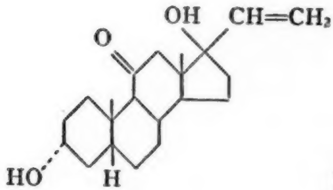

LXVII.



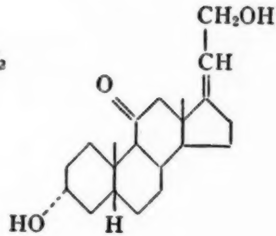
LXVIII.



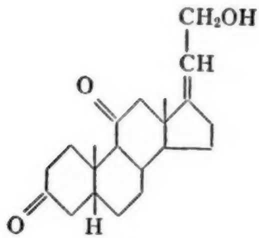
LXIX.



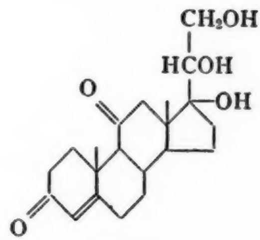
LXX.



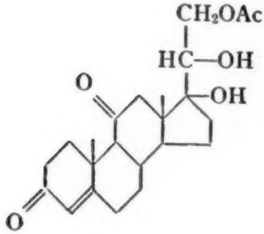
LXXI.



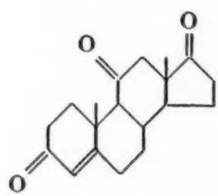
LXXII.



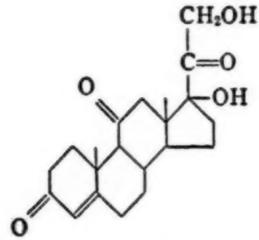
LXXIII.



LXXIV.

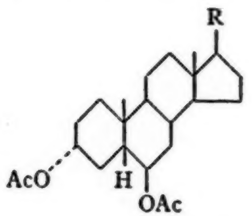


LXXV.

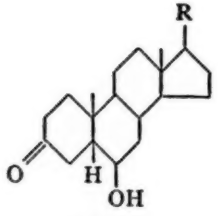


LXXVI.

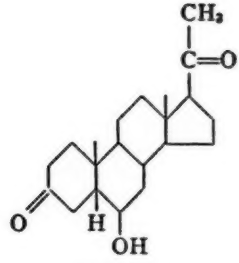
*Miscellaneous steroid transformations.*—The application of oxidation methods to the nucleus of steroids has been extended. Thus, methyl 3(α),6(β)-diacetoxycholanic acid (LXXVII) has been converted (48, 51) into 3-keto-6-hydroxycholanic acid (LXXVIII) by partial saponification, followed by oxidation of the $C_3$ hydroxyl group. The method of partial saponification is similar to that previously described (49) for the preparation of pregnanol-6(β)-dione-3,20 (LXXIX). The tosylate of LXXVIII yields



LXXVII.

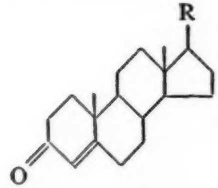


LXXVIII.

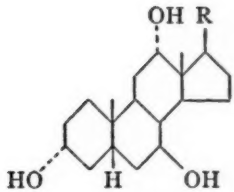


LXXIX.

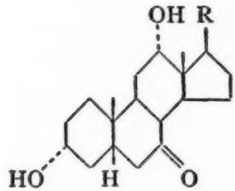
$\Delta^4$-3-ketocholenic acid (LXXX) when refluxed with collidine. It has also been reported (50) that buffered sodium hypobromite can be used to convert cholic acid (LXXXI) into 3,12-dihydroxy-7-ketocholanic acid (LXXXII) in good yield.




LXXX.

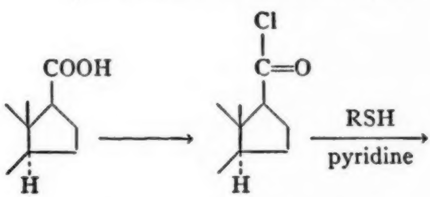



LXXXI.

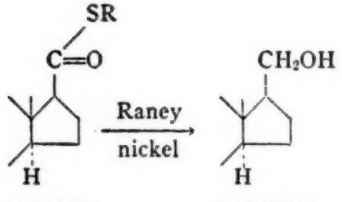



LXXXII.

The application of the reduction of certain types of sulfur compounds with Raney nickel has been extended (52) to the preparation of carbinols from thiol esters. In the steroid series 3($\beta$)-acetoxy*etioallo*cholanic acid (LXXXIII) has been converted to the *etio*cholanylcarbinol (LXXXVI). It has also been reported that $\Delta^5$-3($\beta$)-acetoxy*etio*cholenic acid can likewise be made into the corresponding carbinol. Excellent yields are obtained. The applicability of the Raney nickel hydrogenolysis reaction to steroid thioacetals has also been demonstrated (53).

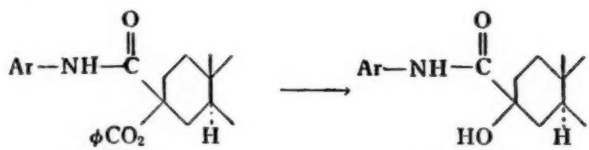



LXXXIII. LXXXIV.



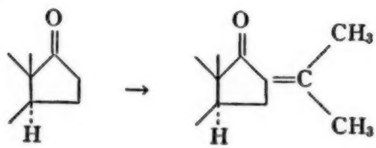
LXXXV. LXXXVI.

Saturated 3-keto steroids have been shown (54) to react with aryl isocyanides in the presence of carboxylic acids to give α-acyloxy-N-aryl amides, LXXXVII. Hydrolysis gives the α-hydroxy compound, LXXXVIII, which, however, cannot be



LXXXVII. LXXXVIII.

further hydrolyzed. Steroids with the keto group in $C_{17}$ have been found to react (55) with acetone in the presence of a base to give 16-isopropylidene derivatives LXXXIX.



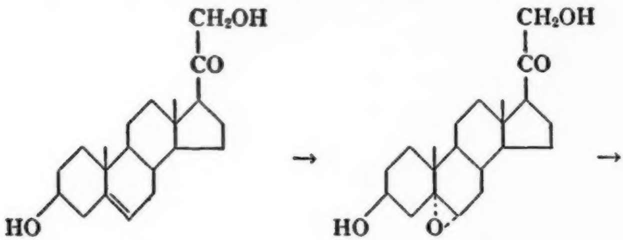
LXXXIX.

Studies carried out on α,β-dibromo compounds and α-bromo ketones in the steroid series indicate that they can be reduced (56) in good yield by chromous chloride to the parent ethylene or ketone respectively. Additional information has been obtained (57) concerning the behavior of certain derivatives of androstenol. Thus, when androsten-5-ol-3(β)-benzoate is heated with benzoquinone a mixture of products is obtained, the ultraviolet ab-

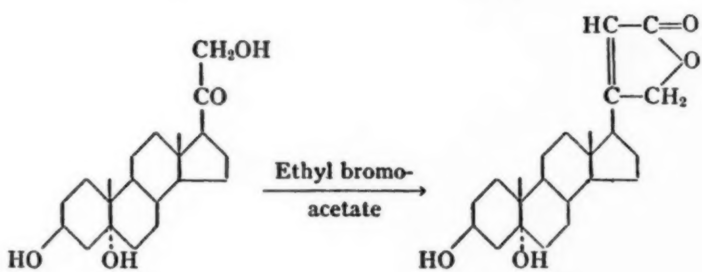
sorption of which indicates a content of 10 per cent of the $\Delta^{5,7}$-diene. After irradiation the mixture was found to be devoid of antirachitic action. Similar results were obtained with androsten-5-diol-3($\beta$),17.

In some earlier experiments (58) it has been reported that androstene-5-ol-3($\beta$)-one-17 can be converted into androstene-5-triol-3($\beta$),16,17. Following the same reaction scheme these studies have been repeated (59), and a triol of different configuration has been isolated. This triol appears to be identical with that isolated from the urine of a patient with an adenocarcinoma of the adrenal cortex.

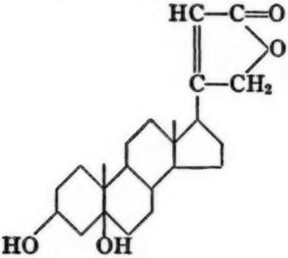
During the past year an approach has been made to the synthesis of digitalis aglucones. A compound, XCIII, isomeric with 14-desoxyperiplogenin (XCIV) has been prepared (60). Catalytic reduction of the $\alpha$-oxide of pregnene-5-diol-3($\beta$), 21-one-20 (XCI) was found to give *allo*pregnanetriol-3($\beta$), 5($\alpha$), 21-one-20 (XCII). Condensation with ethyl bromoacetate produced XCIII.




XC. XCI.

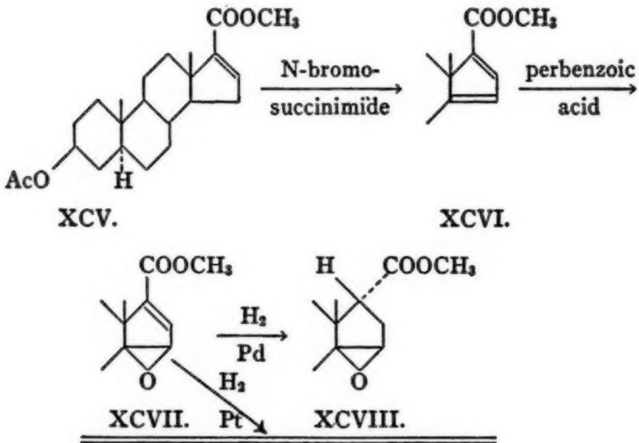


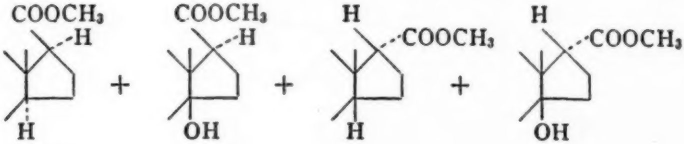

XCII. XCIII.



XCIV.

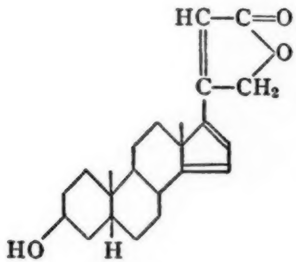
Methods for the introduction of a $C_{14}$ hydroxyl group into the steroid nucleus have been investigated (61). Starting with an unsaturated ester of structure XCV a compound XCVI was prepared by the action of N-bromosuccinimide. With perbenzoic acid this latter compound was converted into a substance of structure XCVII. As a result of configurational studies one is able to assign to the oxide the $\beta$ configuration. Reduction of this oxide gave a saturated oxido ester XCVIII, the configuration of which at $C_{17}$ is $\alpha$. Reduction of XCVII with platinum in alcohol gave four identifiable products, XCIX to CII, two of which have a tertiary hydroxyl at $C_{14}$ with the configurations as indicated.



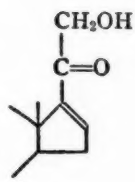


XCIX. C. CI. CII.

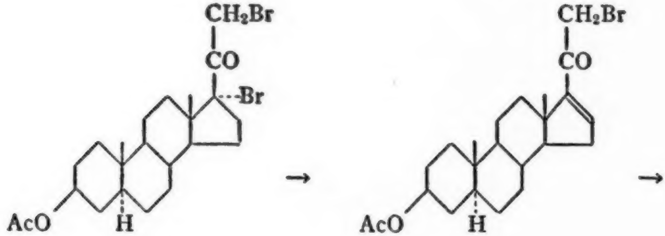
Intermediates for the preparation of compounds of the type of dianhydrogitoxigenin (CIII) require side chains of the type shown in Figure CIV. A variation of a known method (62) was used (63) to prepare compounds of structures CV and CVI. The $\Delta^{16}$-androstenylbutenolide (CVII) has been synthesized by two methods (64) briefly outlined below. Action of a second mole of N-bromosuccinimide on CVII yields the diene CVIII similar to dianhydrogitoxigenin (CIII) in Ring D and in the side chain.



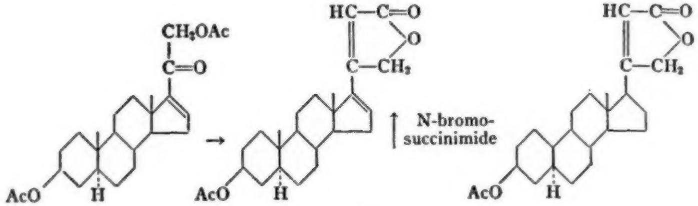
CIII.



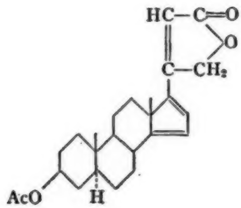
CIV.



CV. CVI.

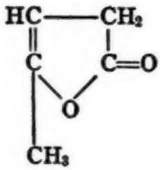


CVII.

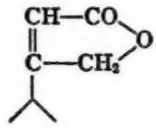


CVIII.

A method for the synthesis of $\gamma$-substituted butenolides of the $\alpha$-angelica lactone type, CIX, has also been developed. The following reaction scheme briefly outlines the details of preparation.

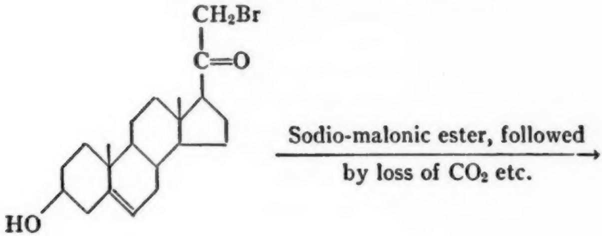


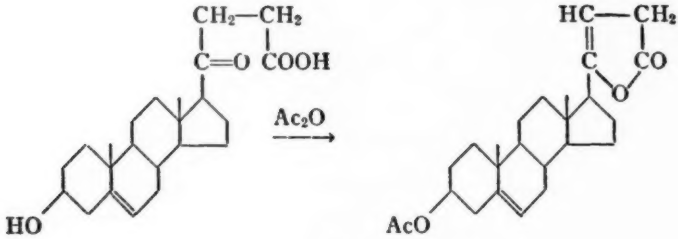
CIX.



CX.

The 3-acetoxyl group in CXI cannot be hydrolyzed, however, without opening the lactone ring. Thus, this ring is more sensitive than the ring in the $\beta$-substituted butenolides of type CX.




CXI.

## STEROID METABOLISM

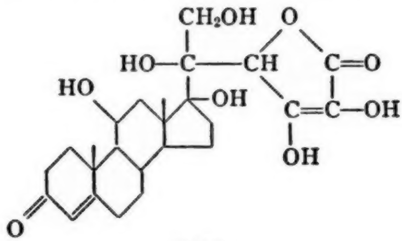
The year has witnessed the publication of many papers in this field. Of special interest to your reviewers are those having to do with the function of the adrenal glands and with the metabolism of 17-ketosteroids.

Following nonfatal hemorrhage it has been reported (65) that both the cholesterol and the ascorbic acid content of the adrenals of a rat are decreased. Hypophysectomy prevents these alterations in adrenal cholesterol and in ascorbic acid after bleeding. Shock induced in rats by hemorrhage has been found to be associated with marked depletion of adrenal cholesterol. The cholesterol content of the liver and brain, however, is unaffected. Plasma cholesterol falls during shock. These alterations suggest an association with the excretion of the cortical hormones, and a participation in their formation.

The effects of temperature and pressure have been studied (66), and it has been reported that the adrenal cholesterol of adult male rats falls to half normal levels as a result of exposure to barometric

pressures of 200 to 300 mm. Rats subjected to temperatures of 0°C. to 5°C. for sixteen to twenty-two hours suffer a small loss of adrenal cholesterol. If exposed to cold for seventy-two hours the adrenal cholesterol returns to normal. Diethyl stilboestrol administration induces a rapid and progressive loss of cholesterol from the adrenal glands.

The isolation of a new active steroid from the adrenal cortex has been announced (67). Its empirical formula has been determined and has been found to be $C_{25}H_{34-36}O_9$. On mild hydrolysis in the absence of air ascorbic acid has been isolated. Oxidation with periodic acid or chromic acid gives adrenosterone (LXXV). The following structure CXII has been proposed.



CXII.

Certain results of studies on the isolation of steroids from pathogenic urine should be noted. A new steroid, $C_{21}H_{34}O_3$, of melting point 219°C. has been obtained (68) from the urine of a woman with adrenal hyperplasia, also from the urines of a cryptorchid male, of a woman with an adrenal tumor, and of a eunuchoid male given testosterone by injection. Structural studies have shown it to be pregnanediol-3($\alpha$),17-one-20. The position of a hydroxyl group at $C_{17}$ suggests an adrenal cortex origin.

The presence of $\Delta^5$-pregnenediol-3($\beta$),20($\alpha$) has been demonstrated (69) in the urines from a woman suffering from an adrenal cortical cancer, and from a girl showing signs of virilism. In the former case the concentration, twenty weeks before death, was 0.33 mg. per l.; two weeks before death it had risen to 24.5 mg. per l. In the latter case 0.18 mg. per l. was the average concentration. The presence of $\Delta^5$-androstenediol-3($\beta$)-17($\alpha$) was also demonstrated in the urine of the patient suffering with adrenal cortical

cancer. In a similar carcinoma (70) an examination of the nonketonic fraction precipitable with digitonin showed the presence of five crystalline steroids, $\Delta^5$-pregnenediol-3($\beta$):20($\alpha$), $\Delta^5$-androstenediol-3($\beta$),17($\alpha$), a triol, $C_{21}H_{34}O_3$, and two undetermined crystalline products. Other compounds which have been isolated (71, 72, 73) from the urine of such patients include a new 17-ketosteroid, androstanediol-3($\alpha$),11-one-17, two isomeric diols, $C_{19}H_{32}O_2$, pregnanediol-3($\beta$),20($\alpha$), pregnanetriol-3($\alpha$),17,20, and an androstenol-3($\alpha$)-one-17.

Results of studies on the metabolism of certain other steroids should be noted. The isolation of dehydro*iso*androsterone from the urine of male guinea pigs, following the administration of $\Delta^5$-androstenediol-3($\beta$),17($\alpha$), has been reported (74). When androsterone is administered (75), either orally, or subcutaneously, *iso*androsterone is excreted.

The metabolism of testosterone in humans has been studied (76), and it has been found that, following the administration of testosterone propionate to a normal female, androsterone, *etio*cholanol-3($\alpha$)-one-17, and $\Delta^2$- or $\Delta^3$-androstenone are excreted in the urine. The percentage recovery of urinary metabolites of testosterone is greater from women than it is when administered to men. A small quantity of a nonketonic substance, androstanediol-3($\alpha$),17($\alpha$) was also obtained. Following administration of dehydro*iso*androsterone to a man, androsterone, *etio*cholanol-3($\alpha$)-one-17, and $\Delta^5$-androstenediol-3($\beta$),17($\alpha$) were isolated (77).

The rate of metabolism of such steroid hormones as testosterone and estrone by the livers of different species has been studied (78). Incubation experiments with the liver mince of the human, the rat, the mouse, the rabbit, and the dog, have shown that the rate of destruction of these hormones is the slowest in human liver, and the most rapid in the liver of the rat and the mouse. Also it should be noted that there appears to be no correlation between the rates of destruction of estrone and of testosterone. It has also been shown (79) in a striking manner that the liver inactivates urinary estrogens even when enormous fibromatogenic quantities are absorbed from the spleen and when administration is continued for several months. The inactivation, however, is not always complete and the question is still open as to the special changes the hormones undergo.

Interesting experiments have been carried out on the synthesis of steroids by the liver (80). When slices of rat liver are incubated aerobically in a phosphate buffer in the presence of deuterium oxide and deuteroacetate containing the isotype $C^{13}$ in the carboxyl group, the cholesterol isolated contains both deuterium and $C^{13}$ in its molecular structure. This fact demonstrates that the liver synthesizes cholesterol *in vitro*. No such syntheses take place, however, under anaerobic conditions.

In concluding this section the following results of isolation studies should be noted. It has been reported (81) that horse testes contain small amounts of testosterone. Hitherto this hormone has been isolated only from the bull.

From pregnant mare's urine two new compounds have been obtained (82), both of which appear to be sulfates. In the one case hydrolysis yields a product identical with pregnene-16-ol-3($\beta$)-one-20.

The structure of certain ethyl esters isolated (83) from the fresh bile of the cow indicates that the bile contains small amounts of 7,12-dihydroxy-3-keto and 3,12-dihydroxy-7-ketocholanic acids, either in a free or conjugated state.

The isolation of $\alpha$-estradiol from the urine of stallions has been reported (84). The concentration appears to be 57000 rat units per l. This is the richest source of $\alpha$-estradiol discovered to date.

Isolation experiments carried out (85) on the normal urine of man indicate the presence of androstanediol-3($\alpha$), 11-one-17 (0.3 mg. per l.). (Note that this is considerably less than the amounts isolated from the urine of patients suffering with an adrenal cortical tumor.) The urine of normal males contained the equivalent of 40 to 85 $\mu$g. of 17-hydroxy-11-dehydrocorticosterone per twenty-four hours (86). In normal adult females the amount excreted is 25 to 65 units. No corticoids were found in infants one to four days old. However, at ages two to three years the value was found to be 35 to 42 units.

A fractionation of hog adrenal cortex extract has been made (87) and it has been reported that there is present a high concentration of 11-oxygenated steroids—six times as much 17-hydroxycorticosterone as in beef adrenals. There is also present after the removal of the 11-oxygenated steroids a water-soluble fraction of relatively low activity by the work test, and high ac-

tivity by the growth survival test. Its activity does not seem to be caused by any of the known active adrenal steroids.

Investigations on urine of pregnancy have been continued (88, 89). It has been shown that in six different samples of supposedly pure pregnanediol glucuronidate, a water-soluble derivative of pregnanol-3($\alpha$)-one-20 is also present. This may account for the low yields in the acid hydrolysis of pregnanediol glucuronidate obtained by several groups of workers.

*Steroid analysis.*—The past two years have witnessed continued progress in this field. A new method (90) for the quantitative estimation of corticosteroids in urine has been announced. It is based on the determination of formaldehyde after periodate oxidation of the urinary extract and it determines the amounts present of both the active and inactive compounds with hydroxyl groups at $C_{21}$ and oxygen at $C_{20}$.

It has been observed (92) that the water-soluble condensation products between 17-ketosteroids and Girard's Reagent T show a characteristic polarographic wave. A technique for the quantitative estimation of 17-ketosteroids has now been evolved (91) based upon this fact.

An analysis has been made (93, 94) of the structure of various steroids by the use of infrared measurements. It is to be noted in this connection that although many absorption bands are not assignable as yet to specific groups they are still valuable for identification. Four bands characteristic of the benzenoid ring are very helpful in differentiating between estrogens and other types of steroids. Spectrophotometric determination (95) of the 2,4-dinitrophenylhydrazones of estrogens also permits estimation of the amounts present.

A new colorimetric assay of urinary corticosteroids has been described (96). By this method only 11-oxycorticosteroids with a 17-hydroxyl group are determined essentially quantitatively. Preliminary results on both normal and abnormal subjects indicate that the substances measured are an index of the rate of secretion of those adrenal cortical hormones which influence protein and carbohydrate metabolism.

A study has been made (97) of the fractionation of neutral urinary steroids by adsorption on magnesium oxide, and elution with ether. This method has also been compared with the Girard

separation method. In the *m*-dinitrobenzene reaction the absorption curves are more nearly like that of androsterone than are those of the ketonic fractions. They also follow Beer's law more closely. Absorption curves of the colored compounds formed from magnesium oxide eluates and of the ketonic fractions with the antimony trichloride reagent show a similarity both to each other and to the crystalline 17-ketosteroids.

Certain new chromatographic separations of steroids have been made (98, 99). The 2,4-dinitrophenylhydrazones of estrone and equilenin can be separated in this manner. The sodium salts of bile acids have been found to give colored esters with *p*-bromomethylazobenzene. A mixture of such esters of cholic and desoxycholic acids have been separated by chromatographing over magnesium carbonate.

A possible method has been described (100) for characterizing cortical hormone metabolites in urine with a two carbon triol or diolone side chain. This method is based on (*a*) periodate cleavage followed by isolation of either the 17-ketone or 17-hydroxy *etio* acid, and (*b*) upon acetal formation with an alkali-soluble aldehyde, *m*-hydroxybenzaldehyde.

It has been reported (101) that the relative amounts of total and of esterified blood cholesterol can be determined without using saponification procedures. This is accomplished by applying a constant correction factor to the cholesterol value obtained by the Liebermann-Burchard color estimation method.

Finally, it should be noted that certain improvements have been made (102) in the method of Schoenheimer and Sperry for the estimation of free blood cholesterol.

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